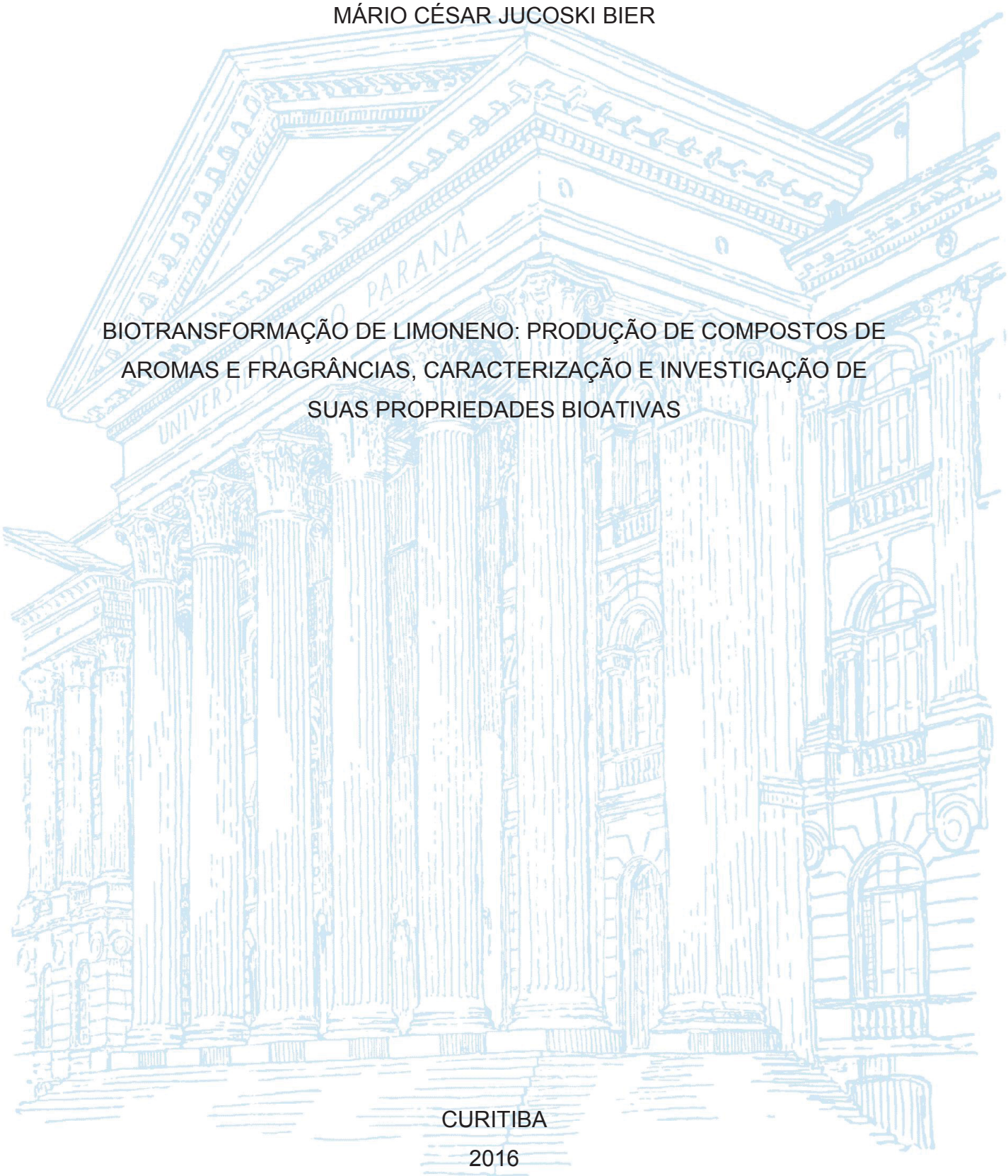
UNIVERSIDADE FEDERAL DO PARANÁ

MÁRIO CÉSAR JUCOSKI BIER



BIOTRANSFORMAÇÃO DE LIMONENO: PRODUÇÃO DE COMPOSTOS DE AROMAS E FRAGRÂNCIAS, CARACTERIZAÇÃO E INVESTIGAÇÃO DE SUAS PROPRIEDADES BIOATIVAS

CURITIBA

2016

MÁRIO CÉSAR JUCOSKI BIER

BIOTRANSFORMAÇÃO DE LIMONENO: PRODUÇÃO DE COMPOSTOS DE AROMAS E FRAGRÂNCIAS, CARACTERIZAÇÃO E INVESTIGAÇÃO DE SUAS PROPRIEDADES BIOATIVAS

Tese apresentada ao Programa de Pós-Graduação em Engenharia de Bioprocessos e Biotecnologia, setor de Tecnologia, Universidade Federal do Paraná, como requisito parcial à obtenção do grau de doutor.

Orientadora: Prof. Dra. Adriane Bianchi Pedroni Medeiros

CURITIBA

2016

FICHA CATALOGRÁFICA ELABORADA PELO SISTEMA DE
BIBLIOTECAS/UFPR BIBLIOTECA DE CIÊNCIA E TECNOLOGIA

B588b Bier, Mário César Jucoski
Biotransformação de limoneno: produção de compostos de aromas e fragrâncias, caracterização e investigação de suas propriedades bioativas / Mário César Jucoski Bier. – Curitiba, 2016.

Tese (Doutorado) - Universidade Federal do Paraná, Setor de Tecnologia, Programa de Pós-Graduação em Engenharia de Bioprocessos e Biotecnologia, 2016.

Orientadora: Adriane Bianchi Pedroni Medeiros.

1. Aromas. 2. Resíduo de laranja. 3. Limoneno-1, 2-diol. 4. Compostos bioativos. 5. Fungos endofíticos. I. Universidade Federal do Paraná. II. Medeiros, Adriane Bianchi Pedroni. III. Título.

CDD: 660.63

Bibliotecária: Romilda Santos - CRB-9/1214

MINISTÉRIO DA EDUCAÇÃO
UNIVERSIDADE FEDERAL DO PARANÁ
PRÓ-REITORIA DE PESQUISA E PÓS-GRADUAÇÃO
Setor TECNOLOGIA
Programa de Pós Graduação em ENGENHARIA DE BIOPROCESSOS E BIOTECNOLOGIA
Código CAPES: 40001016036P8

TERMO DE APROVAÇÃO

Os membros da Banca Examinadora designada pelo Colegiado do Programa de Pós-Graduação em ENGENHARIA DE BIOPROCESSOS E BIOTECNOLOGIA da Universidade Federal do Paraná foram convocados para realizar a arguição da Tese de Doutorado de **MARIO CESAR JUCOSKI BIER**, intitulada: "**BIOTRANSFORMAÇÃO DE LIMONENO: PRODUÇÃO DE COMPOSTOS DE AROMAS E FRAGRÂNCIAS, CARACTERIZAÇÃO E INVESTIGAÇÃO DE SUAS PROPRIEDADES BIOATIVAS**", após terem inquirido o aluno e realizado a avaliação do trabalho, são de parecer pela sua APROVAÇÃO.

Curitiba, 29 de Novembro de 2016.

ADRIANE BIANCHI PEDRONI MEDEIROS
Presidente da Banca Examinadora (UFPR)

CRISTINE RODRIGUES
Avaliador Externo (UFPR)

JULIO CESAR DE CARVALHO
Avaliador Interno (UFPR)

ROSÁLIA RUBEL
Avaliador Externo (IPP)

SANDRO JOSÉ RIBEIRO BONATTO
Avaliador Externo (IPP)

AGRADECIMENTOS

À Universidade Federal do Paraná pela oportunidade cedida para realização deste trabalho.

A minha mãe Teresa Golembioski Jucoski Bier e meu Pai Mário Ildefonso Ribeiro Bier por todo o amor e carinho.

A minha querida esposa Romieli do Rocio Ribeiro por todos os momentos compartilhados nesta trajetória e por todo o apoio.

A minha orientadora Professora Dra. Adriane Bianchi Medeiros pela oportunidade e pela orientação, apoio, ensinamentos valiosos e conhecimentos compartilhados.

Aos professores Dr. Sandro Bonatto e Dra. Rosalia Rubel, assim como a todos os funcionários e colegas do instituto Pelé pequeno Príncipe, em especial a Suzany Hellen e Adriana Yamagushi.

Aos professores doutores Julio Cesar de Carvalho, Cristine Rodrigues, Sven Mangelickx e Norbert D. Kimpe pelas sugestões e contribuições relevantes.

A todos os professores do corpo docente do programa de Pós-Graduação em Engenharia de Bioprocessos e Biotecnologia.

À Coordenação do programa de Pós-Graduação em Engenharia de Bioprocessos e Biotecnologia da UFPR.

Agradecimento aos funcionários que trabalharam nos laboratórios LPBI e LPBII em especial à Mitiyo Miyaoka e Otacilio Pires Tomaz Junior.

Aos amigos e também aos colegas dos laboratórios LPBI e LPBII que dividiram experiências e conhecimento durante o tempo que compartilhamos juntos.

RESUMO

Desde há muito tempo, o aroma ou sabores desempenham papéis importantes na vida humana. Até o século 20, muitos aromas naturais foram obtidos a partir de animais e plantas superiores. A fermentação tem grandes perspectivas de aplicação na alimentação humana e animal como uma técnica para a produção biotecnológica de aromas e fragrâncias. Ela é responsável por mais de um quarto do mercado mundial de aditivos alimentares. Um interesse global em produtos que promovem benefícios para a saúde é crescente e deve aumentar a exigência de alimentos e bebidas, cosméticos e produtos de higiene pessoal. Aromas e fragrâncias são usados com antioxidantes e vitaminas para reduzir os gostos e odores indesejados. Em adição às características sensoriais, existem outras características desejáveis para os compostos de aromas, tais como agentes antibacterianos, antifúngicos e atividade antiviral; Atividade antioxidante; Redução de gordura; Regulação da pressão arterial; Propriedades anti-inflamatórias. A presente pesquisa tem como objetivo aumentar as alternativas para a produção e recuperação de aromas, oferecendo rotas biotecnológicas alternativas e a utilização de resíduos agroindustriais líquidos e sólidos. Objetiva estabelecer precedentes do resíduo de laranja para processos de biotransformação de limoneno e confirmar o potencial dos fungos endofíticos na produção de compostos de aroma e compostos bioativos. E, sobretudo, comprovar as propriedades bioativas destes derivados de limoneno, uma vez que este já possui propriedades interessantes. Um processo para a produção de aromas a partir de resíduos sólidos e do extrato de laranja foi desenvolvido com fermentação no estado submerso. Os principais resultados foram à produção de carvona, mentol e limoneno-1,2-diol na fermentação submersa e a produção de limoneno-1,2-diol e α-terpineol na fermentação no estado sólido após cinéticas de produção e otimização. O fermentado e o produto derivado apresentaram toxicidades inferiores a seus respectivos precursores. Apesar das concentrações obtidas não terem sido as maiores registradas, a maior parte dos trabalhos conta com o uso de meios sintéticos. Não há registro até o momento de tais resultados em fermentação no estado sólido ou da biotransformação do limoneno com fungos endofíticos obtendo-se resultados significativos.

Palavras-chave: Aromas, resíduo de laranja, limoneno-1,2-diol, compostos bioativos, fungos endofíticos.

ABSTRACT

Since a long time, the aroma or flavors play important roles in human life. Until the 20th century, many natural scents were obtained from superior plants and animals. The fermentation process has great prospects of application in human and animal food as a technique for the biotechnological production of aromas and fragrances. It accounts for more than a quarter of the world's food additive market. A global interest in products that promote health benefits is increasing and should increase the requirement for food and beverages, cosmetics and personal care products. Aromas and fragrances are used with antioxidants and vitamins to reduce unwanted tastes and odors. In addition to the sensory characteristics, there are other desirable characteristics for flavor compounds, such as antibacterial, antifungal and antiviral activity; Antioxidant activity; Fat reduction; Blood pressure regulation; Anti-inflammatory properties. The present research aims to increase the alternatives for the production and recovery of aromas, offering alternative biotechnological routes and the use of liquid and solid agroindustrial residues. It aims to establish precedents for the orange residue in the biotransformation process of limonene and to confirm the potential of endophytic fungi in the production of aroma compounds and bioactive compounds. And, above all, to prove the bioactive properties of these limonene derivatives, since it already has interesting properties. A process for the production of flavors from solid residues and orange extract was developed with fermentation in the submerged state. The main results were the production of carvone, menthol and limonene-1,2-diol in submerged fermentation and the production of limonene-1,2-diol and α-terpineol in solid-state fermentation after production kinetics and optimization. The fermented product and the derived product showed toxicities inferior to their respective precursors. Although the concentrations obtained were not the highest recorded, most of the studies rely on the use of synthetic media. There is no record to date of such solid fermentation or biotransformation results of limonene with endophytic fungi obtaining significant results.

Keywords: Aromas, orange residue, limonene-1,2-diol, bioactive compounds, endophytic fungi.

LISTA DE FIGURAS

Capítulo 1 – Revisão Bibliográfica

Capítulo 2 – Liquefied gas extraction: A new method for the recovery of terpenoids from agroindustrial and forest wastes

Capítulo 3 – Biotransformation of limonene by an endophytic fungus using synthetic and orange residue-based media

Capítulo 4 – Biotransformation of limonene in solid state fermentation of orange waste, antioxidant activity and in vitro cytotoxicity of the fermentation extract

Capítulo 5 – Immunomodulatory and antitumoral effects of orange waste fermented by the endophyte *Phomopsis sp.*, orange extract, limonene and limonene-1,2-diol

LISTA DE TABELAS

Capítulo 1 – Revisão Bibliográfica

Capítulo 2 – Liquefied gas extraction: A new method for the recovery of terpenoids from agroindustrial and forest wastes

Capítulo 3 – Biotransformation of limonene by an endophytic fungus using synthetic and orange residue-based media

Capítulo 4 – Biotransformation of limonene in solid state fermentation of orange waste, antioxidant activity and in vitro cytotoxicity of the fermentation extract

Capítulo 5 – Immunomodulatory and antitumoral effects of orange waste fermented by the endophyte *Phomopsis sp.*, orange extract, limonene and limonene-1,2-diol

SUMÁRIO

INTRODUÇÃO

Aromas ou fragrâncias são extremamente importantes para as indústrias de alimentos, rações, cosméticos, químicos e fármacos. Atualmente, flavors representam mais de um quarto de todo o mercado mundial de aditivos alimentares e a maior parte deles provêm de fontes naturais e os métodos tradicionais como síntese química (Akacha e Gargouri, 2014).

É apontado por vários autores que as frutas cítricas possuem altas concentrações de limoneno (Arce *et al.,* 2007, Diaz *et al.,* 2005; Yadava *et al.,* 2004; Fisher e Phillips, 2008) e outros terpenos. Esta razão torna as frutas cítricas e seus resíduos ótimos substratos para o processo de biotransformação do limoneno. De acordo com Rezzadori *et al.,* (2012) o resíduo de laranja pode ser utilizado maneiras diversas. As mais notáveis incluem produção de bio-óleo e carvão vegetal, extração do óleo essencial, produção de pectina, uso como adsorvante e processos para produção de biogás e etanol. Não há registro, entretanto, de processos de biotransformação utilizando diretamente o resíduo sólido.

Monoterpenos como α-pineno e limoneno são matérias-primas baratas para processos de biotransformação. As biotransformações fúngicas destes precursores naturais para compostos de aroma mais valiosos oferecem uma alternativa interessante para flavors naturais. Terpenos geralmente são isolados de óleos essenciais de muitas plantas e são geralmente baratos (Akacha e Gargouri, 2015). Devido às pesquisas contínuas sobre os compostos terpênicos, numerosos avanços foram feitos em diversos aspectos, incluindo avanços na abordagem das extrações e processamento, análise da estrutura molecular, uso para enriquecimento de sabor, e a mais importante, suas potenciais aplicações farmacêuticas (Hu, 2014).

O potencial de fungos para produzir sabores é de interesse considerável no que diz respeito à sua utilização biotecnológica, uma vez que o presente fornecimento de flavors é principalmente limitado à capacidade de biossíntese das plantas. Fontes vegetais dependem fortemente de fatores que são difíceis de controlar, como a influência do tempo, o risco de doenças a plantas, instabilidade sociopolítica das principais áreas

fornecedoras, e restrições comerciais. A produção biotecnológica de flavors pode representar uma alternativa vantajosa a fontes já existentes (Abraham e Berger, 1994).

Plantas e micro-organismos são os principais recursos que dispomos em nossa caminhada rumo ao descobrimento de novos compostos de importância farmacológica com menor toxicidade. Fungos endofíticos, um grupo extenso e diversificado, possui alta versatilidade metabólica (Deepika *et al.,* 2016). O termo endófito se aplica a fungos capazes de ocupar os tecidos vegetais de forma assintomática e aparentemente saudável (Mueller *et al.,* 2004). O potencial de endófitos, particularmente de fungos endofíticos, na produção de compostos farmacêuticos têm sido bastante explorado. Entretanto, estas descobertas não foram ainda traduzidas em processos industriais para a produção comercial de biofármacos utilizando fungos endofíticos (Kusari *et al.,* 2014).

Na composição dos extratos fermentados obtidos de laranja há como produto principal o limoneno-1,2 diol e como composto de maior concentração, o limoneno preexistente como substrato de fermentação. Outros compostos são evidenciados como metabólitos minoritários, dentre eles destacam-se o α-terpineol, o mentol, o 4-terpineol e a carvona. O limoneno-1,2-diol é um composto que já teve sua aplicação reportada em alimentos, porém tem suas propriedades e toxicidade pouco estudada (FDA, 2015). Por este motivo é de interesse neste projeto a realização de testes de toxicidade e avaliação in vitro de suas propriedades benéficas.

OBJETIVO

O presente estudo tem como foco a produção de derivados de limoneno por biotransformação de substratos de laranja em fermentação submersa e em estado sólido pelo fungo endofítico *Phomopsis sp* e a avaliação das propriedades bioativas destes extratos fermentados, tais como capacidade antioxidante, antitumoral, antimicrobiana e imunomoduladora.

Objetivo geral

Obtenção de compostos de aroma ou fragrâncias a partir da biotransformação dos resíduos da extração de suco de laranja por *Phomopsis sp.*, comprovando suas propriedades benéficas.

Objetivos específicos

- Padronizar o inóculo de *Phomopsis sp*. quanto ao preparo em meio Matsumae, PDA e extrato de laranja.
- Selecionar substratos para a biotransformação do limoneno.
- Desenvolver e selecionar métodos para extração dos produtos resultantes da biotransformação do limoneno.
- Produzir limoneno-1,2-diol e outros derivados do limoneno por fermentação em estado sólido.
- Produzir carvona, mentol, limoneno-1,2-diol e outros derivados do limoneno por fermentação submersa.
- Comparar processos de biotransformação em batelada única e batelada alimentada.
- Otimizar variáveis físicas dos processos fermentativos para produção de compostos de interesse.
- Avaliar a toxicidade e propriedades benéficas tais como atividade antitumoral, antioxidante, antimicrobiano e imunomoduladora dos compostos produzidos.

Capítulo 1 – Revisão Bibliográfica

1. Resíduo de laranja

A previsão global para a produção de laranja do mundo foi estimado em 45,8 milhões de toneladas, enquanto a previsão global para a produção de suco de laranja para 2015/16 foi estimada em 1,6 milhões de toneladas métricas (United States Department of Agriculture, 2016). A associação nacional do exportadores de sucos (CitrusBR) tem como estimativa que a produção total de suco de laranja deve alcançar 810,7 mil toneladas de suco de laranja congelado equivalente na safra de 2015/2016 no Brasil (Netto, 2015). De acordo com Crizel *et al.,* 2013; Mahmood *et al.,* 1998; Fiorentin *et al.,* 2010) o resíduo de laranja no processamento do suco, composto por semente, polpa e casca e compreende aproximadamente 50% da laranja.

A biotransformação do limoneno utlizando meio natural é escassa, porém alguns autores conseguiram desenvolvê-la através da extração do óleo essencial e assim alcançaram a produção de derivados do limoneno na fermentação submersa obtendo α-terpineol (Badee *et al.,* 2011) e α-terpineol e álcool perílico (Maróstica Jr. e Pastore, 2007). Porém, não há ainda registro da produção de derivados de limoneno na biotransformação em estado sólido. O uso de resíduo de laranja na fermentação em estado sólido (FES) já foi registrado por Nicolini *et al.,* (1987) para a produção de basidiocarpos de *Pleurotus ostreatus.* Além do uso da casca de laranja após tratamentos como a hidrólise, alguns produtos já foram obtidos com a fermentação deste resíduo. Mantzouridou *et al.,* (2015) produziu seis diferentes ésteres como produtos majoritários da fermentação em estado sólido com levedura, como acetato de isoamlila, hexanoato de etila e octanoato de etila. Yang *et al.,* (2013) otimizou a produção de triterpenóides com a adição de casca de laranja na FES por fungo *Antrodia cinnamoe.* A casca de laranja já foi também utilizada para a produção de biogás e pectina (Pourbafrani *et al.,* 2010) e após pré-tratamentos, para a produção de etanol (Santi *et al.,* 2014).

2. Endófitos

Endófitos são definidos como micro-organismos, incluindo bactérias, fungos e actinomicetos que habitam extra e intracelularmente os tecidos vegetais por todo o seu

ciclo de vida ou parte dele. Endófitos possuem a habilidade de colonizar os tecidos internos de folhas, pecíolos, caules, galhos, cascas, raízes, frutas, flores e sementes, sem causar qualquer dano aparente ou infecção patogênica para suas plantas hospedeiras (Fouda *et al.,* 2015).

Fungos endofíticos são integrantes importantes dos fungos, são cosmopolitas e ocorrem em todas as plantas conhecidas, incluindo uma grande gama de hospedeiros em vários ecossistemas e, portanto, desempenham uma função importante no meio ambiente. Estima-se que existam mais de um milhão de espécies de fungos endofíticos baseando-se na proporção de 1:4 ou 1:5 de fungos em relação a plantas vasculares (Sun e Guo, 2012).

Muitos dos fungos comumente reportados como endofíticos são considerados patógenos minoritários ou secundários por patologistas florestais. Suas ocorrências comuns em tecidos vegetais sadios e doentes ressaltam a incerteza dos limites que separam endófitos dos agentes patogênicos facultativos e latentes (Mueller *et al.,* 2004). Todas as plantas em ecossistemas naturais parecem ser simbióticas com fungos endofíticos. Este grupo de fungos altamente diverso pode ter profundos impactos em comunidades de plantas conferindo tolerância a estresse biótico a abiótico, aumentando sua biomassa e reduzindo o consumo de água. Apesar de mais de 100 anos de estudo terem resultado em milhares de artigos, a importância ecológica destes fungos permanece pobremente caracterizada (Rodriguez *et al.,* 2009).

Sabe-se que fungos representam uma rica fonte de compostos medicinais desde a descoberta da penicilina. Hoje, as drogas derivadas de fungos variam desde antibióticos a imunossupressores. Enquanto plantas permanecem sendo a maior fonte de medicamentos, a cada nova molécula bioativa identificada de uma fonte vegetal, seguem-se registros de ameaça ou até mesmo a extinção de plantas medicinalmente importantes devido à exploração excessiva (Venugopalan e Srivastava, 2015).

Devido ao que parece ser sua contribuição à planta hospedeira, os endófitos podem produzir uma infinidade de substâncias com potencial para a medicina moderna, agricultura e indústria. Novos metabólitos, antimicóticos, imunossupressores e compostos anticâncer são apenas alguns exemplos do que já foi achado após o isolamento, cultivo, purificação e caracterização de alguns endófitos em um passado

recente. São grandes as perspectivas na busca por novas drogas que sejam candidatas efetivas no tratamento de doenças recém desenvolvidas em humanos, plantas e animais (Strobel e Daisy, 2003). Fungos endófitos são fontes potencialmente importantes de produtos naturais para a agricultura, medicina e indústria, com potencial significativo para combater patógenos humanos e vegetais que estão se tornando cada vez mais resistentes aos medicamentos e pesticidas (Mousa e Raizada, 2013).

Tem sido relatado que estes endófitos possuem a habilidade de produzir diversas enzimas extracelulares, incluindo amilase, pectinase, celulase, gelatinase, xilanase e tirosinase (Fouda *et al.,* 2015), compostos fenólicos (Liu *et al.,* 2007), moléculas bioativas de alto valor como paclitaxel, vincristina, vinblastina, camptotecina e podofilotoxina (Venugopalan e Srivastava, 2015), compostos orgânicos voláteis utilizados como flavors (Abrahão *et al.,* 2013), enzimas lignocelulósicas para degradação da madeira (Oses *et al.,* 2006), moléculas bioativas com importância farmacológica (Deepika *et al.,* 2016; Venugopalan e Srivastava, 2016), síntese de compostos hidrocarbonetos complexos (Ahamed e Ahring, 2011).

2.1 Phomopsis

O gênero *Phomopsis* (teleomorfo: *Diaporthe*) compreende microfungos histopatologicamente importantes de distribuição mundial, com diversas associações com hospedeiro. Este grupo de fungos tem ganhado atenção com a descoberta de novos compostos biquimicamente e fisiologicamente ativos e seu uso direto no campo da biotecnologia, na agricultura e na medicina. A alta distribuição e diversidade biológica das species de *Phomopsis* encorajam a avaliação do potencial de aplicações desse fungo (Udayanga et al 2011).

Chapla (2010) Baseando-se em análises de atividade biológica de metabólitos produzidos por *Phomopsis sp.* notou que o fungo demonstrou ser um excelente produtor de metabólitos secundários, uma vez que foram identificadas 15 substâncias e todas apresentam atividade biológica. Diversos outros trabalhos relatam propriedades bioativas deste fungo, como isolamento e caracterização de metabólitos antimicrobianos a partir de *Phomopsis* (Rakshitha *et al.*. 2013), atividade

antimicrobiana e antitumoral de metabólitos de *Phomopsis sp* isolado (Adelin et al. 2011) e atividade antimicótica (Rukachaisirikula *et al.,* 2008).

3. Flavor / aroma

Flavor ou sabor é uma sensação muito complexa composta primariamente de aroma e gosto, mas também complementado por respostas táteis e à temperatura. O gosto é limitado às respostas da língua as sensações salgado, doce, azedo, amargo e umami. A característica mais importante do flavor é o aroma. Normalmente o ser humano pode distinguir entre vários milhares de odores (Reineccius, 1998). De acordo com Heath (1981) a definição básica de flavor foi dada como "qualquer substância que é capaz de transmitir sabor, odor, ou ambos".

O sentido humano do olfato é desencadeada por pequenas moléculas, não-polares para medianamente polares que assimilam-se receptores proteicos do epitélio olfativo. Eles sinalizam frescor, qualidade e autenticidade de um alimento, portanto, guiando nossa escolha de alimentos. Flavors de fontes vegetais ocorrem como misturas complexas com concentrações muito diferentes, variando geralmente de baixas a concentrações traço (Berger, 2014). O aroma final é uma complexa combinação de diferentes moléculas; Contudo, uma substância usualmente predomina, como a vanilina (baunilha) e benzaldeído (amêndoa). Nas bananas, por exemplo, há diversos compostos que formam o aroma final, porém o acetato de etila e o acetato de isoamila são responsáveis pelo maior impacto no aroma (Soccol *et al.,* 2008).

O tamanho global do mercado de flavors e aromas foi estimado em $8,53 bilhões de dólares em 2015 e estima-se que tenha uma taxa de crescimento anual composta de 3.5% e alcance um valor próximo de 10 bilhões de dólares até 2020 (Mordor Intelligence LLP, 2016).

As informações disponíveis a respeito dos mercados nacionais de aromas e fragrâncias são escassas ou desatualizadas. Os mercados nacionais de aromas, sabores e de fragrâncias movimentaram em 2012, 1,2 bilhão de dólares no Brasil. O subsegmento de fragrâncias se mostra mais expressivo, com faturamento de 700 milhões de dólares em 2012 enquanto o de aromas e Sabores obteve um faturamento de 500 milhões

de dólares em 2012 e deve crescer de maneira acelerada, com crescimento anual médio de 6,1% até 2022 (Bain & Company, 2014).

3.1 Aromas obtidos via biotecnologia

Fungos produzem várias misturas de compostos orgânicos voláteis que se difundem pela atmosfera e solo. Pesquisadores detectaram e caracterizaram diversos perfis de compostos orgânicos voláteis de fungos, muitos dos quais possuem odores característicos e são produzidos durante o metabolismo primário e secundário (Morath *et al.,* 2012). Estes compostos orgânicos voláteis ocorrem como misturas de hidrocarbonetes, aldeídos, cetonas, álcoois, terpenos, fenóis, derivados destes grupos, entre outros.

De acordo com a legislação europeia (EG 1334/2008) um flavorizante classificado como "natural" é um composto obtido por métodos físicos, enzimáticos ou processos microbiológicos adequados a partir de material vegetal, animal ou de origem microbiana. Nos Estados Unidos o código de regulamentação federal (CFR – title 21) da FDA contém uma definição similar, incluindo o termo fermentação (Berger, 2014).

Houve muita evolução desde os flavor formados por microflora de alimentos fermentados tradicionalmente até as cepas de procariotos e eucariotos geneticamente modificadas em biorreatores totalmente controlados (Berger, 2014). Os flavors produzidos de micro-organismos competem com as fontes tradicionais. O *screening* para cepas de produção elevada, a elucidação de rotas metabólicas e precursores e a aplicação da bioengenharia convencional resultou em mais de 100 compostos de aroma derivados da via biotecnológica de produção (Berger, 2009).

Com o desenvolvimento de modernas técnicas analíticas no século 20, o isolamento, separação cromatográfica e identificação estrutural de voláteis se tornou rotina, formando a base para a elucidação da geração de flavors microbianos . Pesquisas nas últimas décadas levaram a grandes avanços no conhecimento da produção microbiana e enzimática de flavors, a qual tem sido frequentemente revista (Shrader, 2007).

Apesar de muitos processos biotecnológicos terem sido registrados, a maioria deles não foi industrialmente aplicada para a produção de aromas e fragrâncias. A maior razão para isso são os baixos rendimentos. Os flavors estão geralmente presentes em baixas concentrações nos caldos fermentados, resultando em altos custos para processos de separação e purificação. Ainda assim, este fato é compensado pelos altos preços dos compostos naturalmente produzidos, os quais costumam ser 10 a 100 vezes mais que os sintéticos (Soccol *et al.,* 2008).

Três características da maioria dos processos biotecnológicos devem ser consideradas para a produção em escala industrial: Reações biocatalíticas são geralmente altamente seletivas, os processos se iniciam com matérias-primas naturais e fontes renováveis, e são processos ecologicamente sustentáveis que não agridem o meio ambiente (Shrader, 2007). Devido à evolução do conhecimento e dos resultados das pesquisas, atualmente, a biotecnologia de flavors é parte integrada da indústria de produção de aromas.

4. Fermentação

A fermentação é o processo pelo qual micro-organismos catalisam reações, sintetizam metabólitos secundários e completam outras atividades fisiológicas sob condições aeróbicas ou anaeróbicas. Durante o processo, os micro-organismos desejados ou metabólitos microbianos acumulam-se. Contudo, há três elementos principais nos estudos de fermentação: o produto alvo, a cepa produtora e o meio fermentativo e suas condições (nutrientes, temperatura, umidade, oxigênio, etc.) (Chen, 2013).

A fermentação tem sido amplamente utilizada para a produção de uma grande variedade de substâncias. Ao longo dos anos, as técnicas de fermentação ganharam grande importância devido as suas vantagens econômicas e ambientais. Técnicas antigas foram posteriormente modificadas e refinadas para maximizar sua produtividade. Isto também envolveu o desenvolvimento de novos processos e equipamentos. (Subramaniyam e Vimala, 2012). Duas técnicas de fermentação podem ser destacadas no presente estudo: Fermentação submersa (FS) e fermentação no estado sólido (FES).

4.1 Fermentação submersa

A fermentação submersa utiliza substratos com água fluindo livremente, como melaços e caldos. Os compostos são secretados no caldo de fermentação e os substratos costumam ser utilizados de forma muito rápida. Esta técnica é geralmente mais indicada para micro-organismos como bactérias, que precisam de meios com alta concentração de umidade. Uma vantagem adicional desta técnica é o fato do processo de purificação ser mais simples (Subramaniyam e Vimala, 2012).

4.2 Fermentação no estado sólido

A fermentação no estado sólido é geralmente definida como o crescimento de micro-organismos em material sólido na ausência ou quase-ausência de água livre. No recente período, a FES demonstrou-se muito promissora no desenvolvimento de diversos bioprocessos e produtos (Pandey *et al.,* 2008). A FES é um sistema de três fases que consiste numa fase gasosa contínua, em um filme líquido e na fase sólida. (Chen, 2013).

Embora a fermentação em estado sólido tenha provido muitos produtos de uso diário para os seres humanos, esta constitui apenas uma pequena parte da indústria de fermentação. Se comparado à fermentação líquida, a eficiência de transferência de calor é baixa, os parâmetros são difíceis de monitorar e controlar, e a concepção e aplicação de biorreatores são difíceis (Chen, 2013).

Em adição ao substrato, há diversos fatores importantes que afetam o processo de fermentação no estado sólido. Estes incluem a seleção de micro-organismos aptos, otimização das variáveis do processo (físicas, químicas e bioquímicas) e isolamento do produto. Baseando-se no requerimento mínimo de atividade de água, fungos e leveduras são micro-organismos mais comumente utilizados na FES (Akacha e Gargouri, 2015).

O estabelecimento das relações entre a fisiologia dos micro-organismos e os fatores físico-quimicos são o foco do desenvolvimento dos processos de fermentação. Estes fatores incluem temperatura, pH, aeração, atividade de água e umidade, natureza do substrato sólido utilizado, etc. Dentre estes, a natureza do substrato e a umidade do meio são os que mais afetam a FES (Singhania et al. 2009). O micro-organismo

desejado deve crescer em condições que favoreçam a síntese do composto desejado. (Akacha e Gargouri, 2015).

A fermentação no estado sólido possui uma série de vantagens e desvantagens devido as suas características físico-químicas como baixa atividade de água e formação de gradientes significativos de temperaturas, nutrientes e produtos. Ela difere qualitativamente do método convencional de fermentação submersa no que diz respeito à esporulação e produção de enzimas e metabólitos secundários (Viniegra-Gonzàlez. 1997). A fermentação no estado sólido é mais efetiva em diversos aspectos, incluindo a maior produtividade, estabilidade dos produtos, baixos custos de produção, baixos custos energéticos e baixa demanda de água. Devido a FES ocorrer a baixas atividades de água, contaminações por bactérias e leveduras são minimizadas. Ademais, micro-organismos envolvidos na FES costumam ter um potencial metabólico maior, uma vez que eles crescem em um meio mais próximo do seu natural (Akacha e Gargouri, 2015).

Recentemente, a FES tem tidocredibilidade em indústrias biotecnológicas devido às potenciais aplicações de metabólitos biologicamente ativos, além de ração, combustíveis, alimentos, químicos e fármacos. A utilização de resíduos agroindustriais como substratos de fermentação provê uma alternativa para o uso destes materiais e agrega valores a estas matérias-primas pouco exploradas (Singhania *et al.,* 2009; Chen, 2013).

5. Biotransformação

Bactérias e fungos sintetizam e degradam um vasto número de compostos naturais e xenobióticos. Biotransformações e bioconversões ocorrem se uma estrutura simples é alterada por oxiredução, hidrólise ou reação de adição, ou por uma sequência destas reações. Processos biocatalíticos possuem duas vantagens com relação a quimiossíntese: Eles funcionalizam carbonos quimicamente inertes, modificam uma funcionalidade em uma molécula multifuncional seletivamente ou especificamente, introduzem quiralidade, resolvem racematos e operam em condições ambientais. (Berger *et al.,* 1999). Estas características são amplamente benéficas a processos de biotransformação de aromas, no entanto o processo de biotransformação de terpenos ainda apresenta muitas limitações.

Os métodos biotecnológicos para a produção de flavors podem ser divididos em dois grupos: Síntese *de novo* e biotransformação. As fermentações envolvem a síntese *de novo*, utilizando micro-organismos que crescem em substratos baratos como açúcares. Exemplos de flavors produzidos em escala comercial desta maneira são citrato e butanoato. (Berger et al. 1999). A síntese *de novo* refere-se à síntese de moléculas complexas a partir de moléculas simples tais como açúcares ou aminoácidos. A biotransformação refere-se ao uso de células microbianas para realizar alterações específicas no produto desejado (Berger, 1999; Akacha; Gargouri, 2015). A biotransformação leva a um produto majoritário específico produzido nas conversões (Akacha; Gargouri, 2015).

6. Terpenos

Tradicionalmente, todos os compostos naturais formados a partir de subunidades de isopreno (CH_2=C(CH_3)-CH=CH), na maior parte provenientes de plantas, são denominados como terpenos (Breitmaier, 2006). É um grupo bastante extenso e diversificado de hidrocarbonetos ao serem modificados podem dar origem a compostos de diversos grupos funcionais, contendo ainda unidades de isopreno, os terpenóides. O termo terpenos se origina de terebintina (lat. *Balsamum terebinthinae*), um líquido obtido da resina dos pinheiros, o bálsamo viscoso de cheiro agradável que flui sobre o corte da casca e a madeira nova de várias espécies de pinheiro (*Pinaceae*).

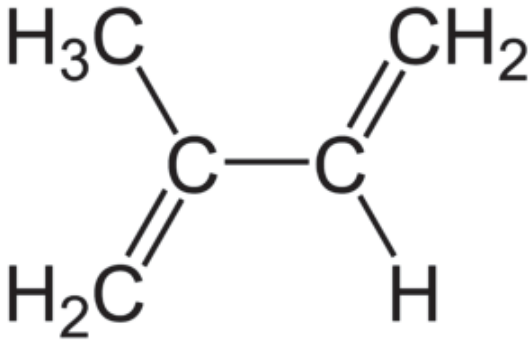


Figura 1: Unidade básica de isopreno

Terpenos e terpenóides são uma grande família de produtos naturais que contam com mais de 50.000 compostos, que apresentam uma diversidade surpreendente no que diz respeito às suas estruturas químicas, suas propriedades físicas e suas atividades

biológicas. Neste contexto, deve-se ressaltar que um número considerável deles é utilizado como ingredientes de aromas e fragrâncias e sua produção representa um recurso importante para as companhias químicas (Serra, 2015).

Muitos dos compostos terpênicos são produtos orgânicos que ocorrem naturalmente em plantas e foram utilizados por séculos como odorizadores devido as suas características aromáticas. Nas últimas décadas tais propriedades têm atraído interesse crescente na área farmacêutica. De fato, um largo número da população mundial utiliza extratos de plantas ou seus compostos ativos por razões ligadas a saúde (Hu, 2014). Madeira de coníferas, bálsamo , frutas cítricas, coentro, eucalipto, lavanda, limão, capim, lírios, cravo, cominho, espécies de hortelã-pimenta, rosas, alecrim, sálvia, tomilho, violeta e muitas outras plantas ou partes desses (raízes, rizomas, caules, folhas, flores, frutos, sementes) são bem conhecidos para cheirar agradavelmente, ter gosto picante, ou exibir atividades farmacológicas específicas. Os terpenos predominantemente são os responsáveis por essas propriedades. (Breitmaier, 2006).

As funções biológicas e ecoquímicas dos terpenos ainda não foram totalmente investigadas. Muitas plantas produzem terpenos voláteis para atrair insetos específicos para a polinização, ou repelir certos animais que as utilizam como alimento. Compostos menos voláteis, porém altamente amargos ou tóxicos também protegem as plantas de serem comidas por animais. Além disso, terpenos desempenham importante função como compostos sinalizadores e reguladores de crescimento (fitormônios) de plantas (Breitmaier, 2006).

A fim de enriquecer os terpenos, as plantas são entalhadas, por exemplo, para a produção de incenso ou óleo de resina das árvores bálsamo; geralmente, os terpenos são extraídos ou destilados por vapor, por exemplo, para a recuperação do óleo das flores de rosas perfumadas. Estes extratos e destilados, conhecidas como óleos etéreos ou essenciais são usados para criar perfumes finos, para refinar o sabor e o aroma dos alimentos e bebidas, e produzir medicamentos de origem vegetal (Breitmaier, 2006)

Enquanto os monoterpenos, seus derivados de diversas funções químicas e sesquiterpenos são amplamente aplicados na indústria como compostos de aromas e fragrâncias, seu precursor, os hidrocarbonetos de terpeno, são geralmente separados de suas fontes naturais, o óleo essencial, pois eles contribuem pouco para aromas e

fragrâncias e também pode ter causar efeitos sensoriais indesejados e precipitações (Schrader , 2007).

6.1 Limoneno

O teor de óleo essencial em plantas é muitas vezes baixo, com concentrações abaixo de 0.1% e a extração comercial de compostos minoritários destes óleos é apenas em casos raros economicamente viáveis. Muitos terpenos como o limoneno e os pinenos são abundantes na natureza, os quais são os principais compostos do óleo de citrus e terebintina, respectivamente. O limoneno, com mais de 90% da composição do óleo de citrus, representa uma matéria-prima ideal para funções biocataliticas que levam a produção natural de aromas e fragrâncias de terpenóides (Berger, 2007).

De acordo com Burdock (2010) o limoneno tem um odor de limão agradável livre das notas de cânfora e terebintina. Ele já foi reportado em mais de 300 óleos essenciais de vegetais variando de 90 a 95% de concentração (limão; laranja) a concentração tão baixas quanto 1% (palmarosa).

O limoneno tem uma infinidade de usos como na manufatura de resinas adesivas, flavors, perfumes, desinfetantes, sabonetes, xampus e soluções de limpeza. Porém, o limoneno pode contribuir drasticamente para a poluição e despejo de resíduos, devido a sua alta demanda química de oxigênio e portanto, é altamente preferível a recuperação e conversão deste composto em um produto mais valioso (NPCS, 2008)

O limoneno é altamente explorado em processos fermentativos devido a seus derivados mais notáveis como α-terpineol, mentol, carvona, limonene-1,2-diol e álcool perílico (Rottava et *al.,* 2011; Bicas *et al.,* 2010; Mukherjee et al., 1973; Vanek *et al.,* 1999) . Ele já foi extensivamente testado quanto as suas propriedades bioativas, as quais costumam ser herdadas por seus derivados e até mesmo potencializadas.

O limoneno e o álcool perílico são os terpenos mais amplamente testados devido as suas propriedades bioativas. Eles já demonstraram benefícios consideráveis em estudos com animais contra uma grande variedade de células malignas. Ambos estão sendo investigados em humanos com câncer avançado com resultados preliminares encorajadores (Murray, 2013).

6.2 α-terpineol

R-(+)-α-terpineol tem um odor tipicamente de Lilás (*Syringa L*). É um dos compostos de aroma mais comumente utilizados. É em sua maioria produzido quimicamente e está comercialmente disponível a baixo custo (Maróstica Júnior e Pastore, 2007).

O α-terpineol é um álcool geralmente estável produzido por síntese química ácido-catalisada a partir de α-pineno ou óleo de terebintina. É um importante produto comercial, normalmente aplicado em sabonetes, cosméticos e preparações de flavors (Bauer et al. 2001). A biotransformação de limoneno para α-terpineol como principal produto já foi descrita por fungos *Cladosporium sp*, *Penicillium digitatum*, *Oxisporum* e *Fusarium*. Para as bactérias, este caminho de conversão tem sido relatado em *Pseudomonas gladiiolos*, um recombinante de *Escherichia coli* e *P. fluorescens* (Bicas *et al.,* 2008).

A produção de α-terpineol é a mais reportada em relação a um metabólito da rota metabólica do limoneno (tabela 1). Já foi reportada sua produção por *Bicas et al.,* (2008) – 2,4 g/L, Tan *et al.,* (1998) – 3,2 g/L, Bicas et al. (2010) – 4,0 g/L, todos em fermentação submersa em meio sintético e por fungos. Adams *et al.,* (2003) obteve a conversão de limoneno por *Penicillium digitatum* após apenas 8 horas. Maróstica e Pastore (2007) atingiram uma concentração de 450 mg/L R-(+)-α-terpineol com *Fusarium oxysporum* 152B /l após 3 dias de fermentação. Ambos os estudos foram realizados, porém, a baixas concentrações de limoneno. Rottava *et al.,* (2011) obteve bons rendimentos na produção de α-terpineol após 144h de fermentação com uma levedura isolada.

Tabela 1: Trabalhos relatando a obtenção do α-Terpineol por biotransformação

Referência	Substrato da biotransformação	Concentração obtida	Tempo de fermentação	Micro-organismo
Maróstica Júnior e Pastore (2007)	Óleo essencial de laranja	450 mg /L	3 dias	*Fusarium oxysporum*
Tan, Day e Cadwallader, (1998)	R-(+)-limoneno	3200 mg/L	1 dia	*Penicillium digitatum*
Bicas *et al.,* (2008)	R-(+)-limoneno	2400 mg/L	3 dias	*Fusarium oxysporum*

Rottava *et al*., (2011)	R-(+)-limoneno	1,700 mg/L	6 dias	Cepa isolada *05.01.35*
Rottava *et al.,* (2011)	(−)-β-pineno	770 mg/L	7 dias	*Aspergillus sp.*
Bicas *et al.*, (2010)	*R*-(+)-limoneno	4.000 mg/L	2 dias	*Fusarium oxysporum*
Tai *et al.,* (2015)	R-(+)-limoneno	833.93 mg/L	12 h	*Penicillium digitatum DSM 62840*

6.3 Limoneno-1,2-diol

Há relatos anteriores de limoneno-1,2-diol ser o produto de biotransformação majoritário em leveduras e fungos filamentosos e um produto minoritário obtido através de biotransformação com linhagens de bactérias. A relação de trabalhos tendo Limoneno-1,2-diol como produto final é relativamente escassa (Tabela 2).

Tabela 2: Trabalhos relatando a obtenção do limoneno-1,2-diol por biotransformação

Referência	**Substrato da biotransformação**	**Concentração obtida**	**Organismo**
Carvalho *et al.,* (2000).	*cis*- epoxido de 1,2-limoneno	94.1% (rendimento)	*Rhodococcus erythropolis*
Mukherjee, Kraidman e Hill (1973).	D-limoneno	1,5 g/L	*Cladosporium sp.*
Molina *et al.,* 2015	S(-) limonene	3.7 g/L	*Fusarium oxysporum 152B*
Demyttenaere *et al.,* 2001	(*S*)-(−)-limoneno	50.49% (yield)	*Corynespora cassiicola*

Limoneno-1,2-diol ou limoneno glicol é um óleo incolor a levemente amarelado com aroma de menta fresca com o consumo ligado ao odor/sabor usado em menta (Burdock, 2010). A produção microbial de limoneno-1,2-diol já foi reportada por alguns autores, usualmente como um produto minoritário da biotransformação do limoneno (Demyttenaere *et al.,* 2001; Carvalho *et al.,* 2003). Entretanto, alguns autores obtiveram limoneno-1,2-diol como o produto majoritário da fermentação de fungos filamentosos em meio sintético. Mukherjee *et al.,* (1973) obteve 1,5 g/L de limoneno-1,2-diol usando *Cladosporium* sp. Recentemente Molina *et al.,* (2015) obteve 3,7 g/L de limonene-1,2-diol usando S(-) limoneno como substrato.

6.4 Carvona

Carvona é descrito por Carvalho e Fonseca (2006) como tendo odor de hortelã doce. Carvona tem grande importância como fragrância e sabor, relevância na área médica e outros aplicações. De acordo com Berger (2007) a carvona é um monoterpeno de cetona importante no qual o (+)-isómero representa o composto de caráter de impacto de sabor alcaravia (até 60 % do óleo de alcarávia), enquanto que o (-)-isomero tem uma nota de hortelã (70-80 % em óleo de hortelã).

Carvona tem sido utilizada como aromatizante de pão (*rye*), queijo, chucrute, doces, carne, molhos e licores. A carvona tem sido utilizada ultimamente, sobretudo nos Países Baixos, no armazenamento em longo prazo de batatas. A carvona impede de forma mais eficaz do que os produtos químicos utilizados habitualmente para que as batatas grelem, ao mesmo tempo em que apresenta uma menor toxicidade para o ser humano. Além disso, este terpeno apresenta propriedades anti-fúngicas e anti-bacterianas (Carvalho e Fonseca, 2006).

Este composto tem usos farmacêuticos e cosméticos. Carvona e plantas ricas neste monoterpeno são amplamente utilizados em alimentos tradicionais e novos alimentos, incluindo a indústria de goma de mascar. Além disso, carvona tem algumas importantes aplicações na agricultura, tanto para a proteção das culturas e como um agente de germinação durante o armazenamento de tubérculo (Morcia *et al.,* 2016)

Poucos trabalhos registraram a produção de carvona (Tabela 3). A produção biotecnológica de carvona usando limoneno como precursor já foi descrita em alguns estudos (Carvalho e Fonseca, 2003; Vanek *et al.,* 1999; Duetz *et al.,* 2001; Trytek e Fiedurek, 2002).

Tabela 3: Biotransformação para produção de carvona

Referência	Substrato da biotransformação	Concentração obtida	Tempo de fermentação	Organismo
Vanek, Valterova e Vaisar (1999)	(*S*)-(−)-limoneno	34% - 24 mg	10 dias	*Solanum aviculare (célula de planta)*
Carvalho e	(+)-(*R*)-limoneno	Não estabelecida	5 dias	*Rhodococcus*

Fonesa (2003)				*opacus*
Duetz et al. 2001	D-limoneno	0.08 mM	27 horas	Células de *Rhodococcus opacus* PWD4

7. Propriedades bioativas

Desde a descoberta da penicilina em 1928 e o seu desenvolvimento clínico subsequente em 1940, produtos naturais, em especial antibióticos, proporcionaram grandes benefícios a humanidade. Nestes mais de 85 anos, tais produtos tornaram-se indispensáveis no campo clínico na forma de antibacterianos, antifúngicos, antiparasíticos, agentes imunossupressores e antitumorais e também altamente essenciais nos campos da veterinária, bem como na agricultura como aditivos de ração ou para plantas (Olano *et al.,* 2013).

O estudo das atividades bioativas de óleos essenciais é um campo em constante expansão atualmente. Dentre as fontes de óleos essenciais destaca-se o óleo essencial de laranja e a casca de laranja que possui aproximadamente 95% de limoneno (Danielski *et al.,* 2008; Bier *et al.,* 2011). As frutas cítricas possuem elevadas concentrações de limoneno (Arce *et al.,* 2007; Diaz, Espinosa e Brignole, 2004; Yadava *et al.,* 2004; Steinbrecher *et al.,* 1999) Diversos estudos confirmam as propriedades do óleo essencial de laranja e casca, como atividade antifúngica (Velázquez-Nuñez *et al.,* 2013), efeitos anti-inflamatórios (Gosslau *et al.,* 2014), antioxidante (Lu *et al.,* 2012; Chen *et al.,* 2012), antitumoral (Kaur e Kaur, 2015) e inseticida (Ezeonu *et al.,* 2001; El-akhal *et al.,* 2015).

O limoneno e seus derivados já tiveram uma série de propriedades bioativas relatadas: atividade antioxidante e antigenotóxica (Bacanli *et al.,* 2015), efeitos sobre prevenção e melhora de dislipidemia e hiperglicemia em ratos (Jing *et al.,* 2013), inibição de angiogênese, metástase e morte celular em células do câncer de cólon humano através de d-limoneno da laranja (Murthy *et al.,* 2012), efeitos ansiolíticos em camundongos (Lima *et al.,* 2013), atividade inibitória contra parasitas da leishmania *in vitro* e *in vivo* (Arruda *et al.,* 2009), prevenção e tratamento de câncer de mama (Miller *et al.,* 2011) entre outros.

Alguns derivados do limoneno já apresentam suas propriedades bioativas estabelecidas. A carvona apresenta propriedades antifúngicas e antibacterianas (Carvalho e Fonseca, 2006). O álcool perílico (AP) é um monoterpeno isolado dos óleos essenciais de menta, cerejas e sementes de aipo, dentre outras plantas. Estudos em animais mostram que o AP é um agente quimioterápico eficaz na regressão de tumores de mama, pâncreas, fígado e próstata, e agente quimiopreventivo nos tumores de cólon, melanomas e neuroblastomas (Fischer *et al.,* 2005). O α-terpineol, um dos principais derivados do limoneno, também apresenta recentemente estudos apontando atividades anticâncer (Hassan *et al.,* 2010) e antioxidante (Maróstica Júnior *et al.,* 2009).

Na composição dos extratos fermentados obtidos de laranja há como produto principal o limoneno-1,2-diol e como composto de maior concentração, o limoneno preexistente como substrato de fermentação. Outros compostos são evidenciados como metabólitos minoritários, dentre eles destacam-se o α-terpineol, o mentol, o 4-terpineol e a carvona. O limoneno-1,2-diol é um composto que já teve sua aplicação reportada em alimentos, porém tem suas propriedades e toxicidade pouco estudada (FDA, 2013). Por este motivo é de interesse neste projeto a realização de testes de toxicidade e avaliação in vitro de suas propriedades benéficas. O extrato produzido também será avaliado quanto às propriedades bioativas, uma vez que o limoneno e diversos derivados têm sido relatados quanto a prováveis propriedades benéficas de interesse (Gelb *et al.,* 1995; Singh *et al.,* 2010; Murthy *et al.,* 2012).

Devido aos indícios já registrados do potencial do limoneno e do extrato de laranja como antioxidante e agente antitumoral e as novas descobertas com relação aos mecanismos de doença e possíveis soluções terapêuticas, há uma grande prospecção na busca por novos produtos naturais, alimentos e fármacos, inserindo-se este trabalho num importante contexto.

7.1 Atividade antioxidante

O estresse oxidativo é um fenômeno associado a patologia de várias doenças incluindo arteriosclerose, doenças neurodegenerativas como mal de Alzheimer e doença de Parkinson, câncer, diabetes mellitus, assim como desordens psiquiátricas e o processo do envelhecimento. O estresse oxidativo é definido como o desbalanceamento entre a produção de radicais livres e os metabólitos reativos, os oxidantes e seu sistema

de eliminação pelos mecanismos de proteção, os sistemas antioxidantes. Este desbalanceamento causa danos a importantes biomoléculas e órgãos com impactos plausíveis a todo o organismo (Ďuračková, 2014).

Antioxidantes possuem a capacidade de prevenir ou reparar danos causados pela oxidação. Nutrientes que funcionam como antioxidantes incluem vitaminas e minerais. Não nutrientes, fitoquímicos dietéticos conhecidos por exercer ação antioxidante incluem compostos de enxofre, compostos fenólicos, ligninas, carotenóides, betaína, colina, o policosanol, melatonina, γ-orizanol , saponinas e fitatos (Beta e Duodu, 2016).

A importância dos antioxidantes no processamento de alimentos tem aumentado. A sua função tradicional e inibir o desenvolvimento de ranço oxidativo em alimentos a base de gordura particularmente carnes, laticínios, produtos de consumo diário e frituras. Porém, pesquisas recentes sugerem um novo papel na inibição de doenças cardiovasculares e câncer.

De acordo com Bacanli *et al.,* (2015) o limoneno é um dos compostos fenólicos mais comuns encontrados em plantas cítricas. Espécies reativas de oxigênio produzidas ao curso de reações bioquímicas são extremamente reativas e podem causar dano a várias moléculas biológicas como proteínas, DNA e lipídios.

Compostos fenólicos já foram identificados como possíveis antioxidantes, por este motivo eles têm sido usados na indústria de alimentos e na prevenção de doenças que resultam do stress oxidativo (Block *et al.,* 1992; Nakatani, 2000). Por outro lado, é possível que vários compostos fenólicos apresentem propriedades pró-oxidativas em doses elevadas (Wong e Mclean, 1999). Fitoquímicos fenólicos devem promover a saúde, em parte, através do seu efeito antioxidante e de eliminição de radicais livres, protegendo assim os componentes celulares contra danos induzidos por estes radicais (Bacanli *et al.,* 2015).

7.2 Atividade antitumoral

O câncer emergiu como uma das doenças mais alarmantes das últimas décadas pelo mundo. É uma doença multifatorial que contribui para o crescimento descontrolado e invasão das células anormais que levam à formação de tumor. Drogas ou agentes anticancerígenos são compostos usados para tratar as células malignas ou impedir sua proliferação.

A toxicidade de agentes anticancerígenos pode ser atribuída às diferenças entre as células cancerosas quando comparadas as saudáveis. As células cancerosas crescem, se dividem e se espalham indefinidamente. Além disso, possuem a capacidade de invadir outros tecidos e causar danos a órgãos. Os mecanismos pro trás dos sistemas errôneos das células malignas muitas vezes as protegem das células de defesa e quimioterapia. Os agentes anticancer são imperfeitos e não reconhecem as diferenças existentes nas células cancerosas. Eles geralmente podem apenas visar células que se dividam extremamente rápido. Porém, isto geralmente danifica células normais que se reproduzam rapidamente (Gad, 2012)

Os neuroblastomas, tipo de tumor correspondente às linhagens celulares utilizadas nos ensaios antitumoral do presente estudo, são tumores sólidos originários do sistema nervoso simpático, exclusivos da infância (Cartum, 2012). Apesar dos grandes avanços terapêuticos e em técnicas de diagnóstico, esse tumor persiste como um grande desafio para os oncologistas pediátricos.

Ele corresponde ao tumor sólido extracraniano mais comum da infância, representando 8% a 10% de todos os cânceres em crianças menores de 15 anos. Entre os lactentes, corresponde a mais de 50% das neoplasias malignas. A incidência anual varia entre 7 e 12 casos por milhão de crianças até 15 anos, alcançando 7,3 casos/milhão no Brasil (Cartum, 2012).

A quimioterapia continua a ser o tratamento padrão para metástase e câncer avançado. Porém, agentes quimioterápicos comumente usados podem induzir danos a células e tecidos saudáveis. Desta maneira, recentemente, houve um aumento no desenvolvimento de novas e mais eficientes drogas anticâncer que exibam baixa toxicidade e que não sejam afetadas por mecanismos de resistência (da Costa *et al.,* 2015).

A maior parte dos agentes anticancer danifica o DNA diretamente, interferem com o metabolismo do DNA ou segregação cromossômica e são particularmente tóxicos para células em divisão. Apesar de uma quantidade considerável de informações sobre os mecanismos de ação destes compostos estarem disponíveis, as bases moleculares para eliminação de células tumorais de forma seletiva continuam

desconhecidas (Simon *et al.,* 2000). Fontes alternativas podem levar a novos compostos que surjam como uma solução para estes tratamentos e possam oferecer respostas melhores e com menos efeitos colaterais.

A recorrência crescente de tumores em mamíferos e os efeitos colaterais dos agentes quimioterápicos reduz a eficiência clínica de uma larga variedade de agentes antitumorais utilizados atualmente. Por conseguinte, há sempre uma necessidade constante de desenvolver drogas alternativas com os mínimos efeitos possíveis. Uma importante estratégia para o desenvolvimento destas drogas é a busca em fontes naturais. Agentes anticâncer derivados de plantas, bem como seus derivados têm se mostrado bastante eficazes na prevenção e tratamento do câncer (Ali *et al.,* 2012).

Na última década, óleos essenciais estiveram sob estudo com relação a seu uso na terapia do câncer. Além das abordagens tradicionais, vários produtos de plantas classificadas como alcalóides, saponinas, triterpenos, glicosídeos, e polifenóis entre outros têm mostrado propriedades anticancerígenas muito promissores em estudos in vitro e in vivo. Esta é uma área em constante expansão no mundo. (Gautam *et al.,* 2014).

Em experimentos do limoneno como agente terapêutico do câncer humano, Crowell *et al.,* (1994) e Poon *et al.,* (1996) descrevem o limoneno-1,2-diol como um dos principais metabólitos produzidos ao lado de ácido perílico e ácido diihidroperílico. Tais estudos a respeito dos metabólitos formados pelo limoneno sugere a possibilidade da formação destes compostos e sua presença serem os responsáveis pela atividade antitumoral exibida pelo limoneno. Roberto *et al.,* (2009) reportou que o limoneno exerce um efeito bifásico na proliferação celular. O aumento da proliferação celular está relacionada à queda de H_2O_2 através do aumento da atividade de catalases e peroxidases. Ademais, o limoneno protegeu as células contra o stress oxidativo induzido pela adição de H_2O_2.

8. Sistema imune

A imunidade inata é a primeira linha de defesa contra as infecções e, em muitos casos pode eliminar os micro-organismos. Os mecanismos de imunidade inata existem

antes do encontro com micro-organismos, e são rapidamente ativados por eles antes do desenvolvimento das respostas imunes adaptativas. A inibição ou eliminação de qualquer um dos vários mecanismos da imunidade inata aumenta de forma significativa a suscetibilidade a infecções, mesmo quando o sistema imune adaptativo está intacto e funcional (Abbas e Lichtman, 2005).

O sistema imune inato consiste em barreiras epiteliais, células circulantes e proteínas que reconhecem micro-organismos ou substâncias produzidas em infecções e iniciam as respostas que eliminam esses micro-organismos. As principais células efetoras da imunidade inata são os neutrófilos, os fagócitos mononucleares e as células NK (natural killer). Estas células atacam micro-organismos que romperam as barreiras epiteliais e entraram nos tecidos ou circulação. Algumas células, especialmente os macrófagos e células NK, secretam citocinas que ativam os fagócitos e estimulam a reação celular da imunidade inata, chamada inflamação (Abbas e Lichtman, 2005).

A fase efetora da resposta imune é mediada, principalmente, por imunoglobulinas naturais e citocinas. Na imunidade natural, funcionam imunoglobulinas naturais e citocinas, ambas produzidas sem participação do agente infeccioso. Na imunidade adquirida, funcionam imunoglobulinas específicas, monocinas e linfocinas, todas induzidas com a participação do agente infeccioso (Silva e Mota, 2003). As citocinas desenvolvem uma importante tarefa no desenvolvimento, regulação e diferenciação das células imunes. Atribui-se a desregulação da produção de citocinas ou da sua atividade como uma das principais culpadas pelo desenvolvimento de doenças imunes e doenças neoplásicas (Rubel *et al.,* 2010).

Os macrófagos constituem um dos três tipos de fagócitos do sistema imunológico, distribuindo-se amplamente nos tecidos, onde passam a desempenhar um papel crítico na imunidade inata. Tais células são a forma madura dos monócitos, que circulam no sangue e se diferenciam continuamente em macrófagos após migrarem para os tecidos (Janeway *et al.,* 2002).

8.1 Atividade imunomoduladora

A capacidade de imunomodulação por biomoléculas pode ser determinada pela produção de citocinas por células mononucleares do sangue periférico humano (PBMC)

como macrófagos e os linfócitos T e B, assim como pelo estímulo a mitogenicidade dessas células e ativação do sistema imune (Shamtsyan *et al.,* 2004; Schepetkin e Quinn, 2006).

Imunomodulação engloba todas as intervenções que visam modificar a resposta imune. O aumento da resposta imune é desejável para prevenir infecções em estado de imunodeficiência, para combater infecções já estabelecidas e para combater o câncer (Gea-Bancloche, 2006).

Existem várias técnicas coletivamente conhecidas como modulação imune, que podem alterar a expressão das citocinas pelos linfócitos T e macrófagos. Essas envolvem a manipulação do ambiente de citocinas em que a ativação das células T ocorre, ou a manipulação do modo como o antígeno é apresentado, pois se observou que estes fatores influenciam a diferenciação e a função secretora de citocinas das células T ativadas (Janeway *et al.* 2002).

O óxido nítrico (NO), um radical gasoso de curta duração, é um potente metabólito reativo que pode agir como um neurotransmissor e dilatador e, é uma das principais moléculas das células imunes contra células tumorais e patógenos. Entretanto, estudos mostraram que a produção excessiva de NO durante infecções costuma causar dano a tecidos endoteliais. Já foi demonstrado que o NO está envolvido com o colapso vascular, o qual é um grande responsável pela mortalidade (Chi *et al.,* 2003).

A atividade antimicrobiana e citotóxica do NO são aprimoradas por outros produtos dos macrófagos como ácidos, glutationa, cisteína, peróxido de hidrogênio e superóxido. Apesar da saída de NO provavelmente ter evoluído para proteger o hospedeiro de infecções, efeitos supressivos na proliferação de linfócitos e danos a outras células do hospedeiro conferem ao NO a mesma dualidade de proteção e destruição inerente a todos os outros principais componentes da resposta imune (MacMcking *et al.,* 1997).

Dentre as diferentes substâncias liberadas por macrófagos e outras células do sistema imune, H_2O_2, TNF-a e NO desempenham um papel especial não apenas devido

a sua atividade antitumoral, mas também devido a sua capacidade de induzir danos aos tecidos (Rubel *et al.,* 2011).

Macrófagos e outras células do Sistema immune produzem mediadores envolvidos na resposta inflamatória, como citocinas, óxido nítrico (NO), e espécies reativos do oxigênio, incluindo o ânion superóxido (O_2^-) e peróxido de hidrogênio (H_2O_2). Devido aos danos aos tecidos que o aumento do estress oxidativo pode causar, alimentos que apresentam propriedades antioxidantes têm sido extensamente buscados pelas companhias (Rubel et al., 2010).

A habilidade dos macrófagos de produzir espécies reativas do oxigênio (EROs) e óxido nítrico (NO) podem aumentar a sobrecarga oxidativa de lipoproteínas de baixa densidade (LDL) e podem acelerar o processo de arterosclerose (Woo *et al.,* 2005). Considerando estes efeitos, antioxidantes naturais podem se empregados como adjuvantes na prevenção e tratamento de dislipidemia (Rubel *et al.,* 2011).

8.2 Citocinas

Citocinas são moléculas protéicas de baixo peso molecular que regulam a imunidade inata e adaptativa. São sintetizadas por vários tipos celulares e se ligam em receptores específicos, induzindo uma ativação celular (Calich e Vaz, 2009). As atividades biológicas específicas das citocinas podem ser agrupadas em quatro categorias: Mediação da imunidade natural; Regulação da ativação, crescimento e diferenciação de linfócitos; Ativação de células inflamatórias não específicas; Estimulação do crescimento e diferenciação dos precursores dos leucócitos e da medula óssea (Silva e Mota, 2003). As citocinas são produzidas em respostas a micro-organismos e outros antígenos. Diferentes citocinas estimulam respostas diversas das células envolvidas na imunidade e inflamação. As citocinas estimulam o crescimento e diferenciação de linfócitos e ativam diferentes células efetoras para eliminar micro-organismos e outros antígenos. As citocinas são importantes como alvos para antagonistas específicos em numerosas doenças imunes e inflamatórias (Abbas e Lichtman, 2005).

8.3 Ânion superóxido

Ânion superóxido derivado de monócitos contribui para o stress oxidativo em sítios inflamatórios, é necessário para a oxidação do LDL mediada por monócitos e altera as funções celulares como adesão e proliferação. Uma das respostas mais imediatas dos monócitos para a variedade de estímulos de ativação é a produção de potentes radicais livres de oxigênio, o ânion superóxido (Rueda *et al.,* 2013). Evidências indicam que a regulação da produção do ânion superóxido contribui para prevenção da arterosclerose (Cathcart, 2004).

8.4 Peróxido de Hidrogênio

De acordo com os estudos de Tang *et al.,* (2007) o peróxido de hidrogênio (H_2O_2) induz ativa e passivamente a liberação of high mobility group box 1 protein (HMGB1) através de culturas de monócitos e macrófagos de maneira dependente de tempo e dosagem. Em doses não tóxicas, H_2O_2 induz a translocação citoplasmática de HMGB1 e sua liberação. Em concentrações mais elevadas, porém, H_2O_2 exibe citotoxicidade a macrófagos e monócitos. O H_2O_2 também é importante para matar bactérias e prevenir infecções (Cho *et al.,* 2001). Portanto, o controle da concentração de peróxido de hidrogênio é algo de grande importância no funcionamento da resposta imune.

Referências

Abbas, A. K., Lichtman, A. H. Imunologia Celular e Molecular, 5º ed. Rio de Janeiro: Elsevier, 2005, 576 p.

Abraham, B. G. Berger, R.G. Higher fungi for generating aroma compounds though novel biotechnologies, Journal of Agricultural and Food Chemistry 42 (1994) 2344-2348.

Abrahão, M. R. E. Molina, G. Pastore, G. M. Endophytes: Recent developments in biotechnology and the potential for flavor production, Food Research International 52 (1) (2013) 367–372

Adelin, E. Servy, C. Cortial, S. Lévaique, H. Martin, M.T. Retailleau, P. Le Goff, G. Bussaban, B. Lumyong, S. Ouazzani, J. Isolation, structure elucidation and biological

activity of metabolites from Sch-642305-producing endophytic fungus *Phomopsis* sp. CMU-LMA, Phytochemistry 72 (18) (2011) 2406-2412

Ahamed, A. Ahring, B.K. Production of hydrocarbon compounds by endophytic fungi Gliocladium species grown on cellulose, Bioresource Technology 102 (20) (2011) 9718-9722

Akacha, N. B. Gargouri, M. Microbial and enzymatic technologies used for the production of natural aroma compounds: Synthesis, recovery modeling, and bioprocesses, Food and Bioproducts Processing 94 (2015) 675–706

Ali, R. Mirza, Z. Ashraf, G. M. Kamal, M. A. Ansari, S. A. Damanhouri, G. A. Abuzenadah, A. M. Chaudhary, A. G. Sheikh, I. A. New anticancer agents: recent developments in tumor therapy. Anticancer Research 32 (7) (2012) 2999-3005.

Arce, A. Pobudkowskal, A. Rodríguez, O. Soto, O. Citrus essential oil terpenless by extraction using 1-ethyl-3-methylimidazolium ethylsulfate ionic liquid: Effect of the temperature, Chemical Engineering Journal 133 (2007) 213-218.

Arruda, D. C., Miguel, D. C., Yokoama-Yasunaka, J. K. U., Katzin, A. M., Uliana, S. R. B. Inhibitory activity of limonene against Leishmania, Biomedicine & Pharmacotherapy 63 (2009) 643-649

Bacanli, M., Basaran, A. A., Basaran, N. The antioxidant and antigenotoxic properties of citrus phenolics limonene and naringin, Food and Chemical Technology 81 (2015) 160-170

Badee, A. Z. M. Helmy, S. A. Morsy, N. F. S. Utilisation of orange peel in the production of α-terpineol by Penicillium digitatum (NRRL 1202), Food Chemistry 126 (2011) 849–854

Bain & Company. Potencial de Diversificação da Indústria Química Brasileira – Relatório 4 – aromas, sabores e fragrâncias. Rio de Janeiro: Bain & Company, 2014.

Bauer, K. Garbe, D. Suburg, H. Common Fragrances and Flavor Materials. Weinheim: Wiley-VCH (2001) 293 p

Berger, R. G. Biotechnology as a source of natural volatile flavours, Current Opinion in Food Science 1 (2015) 38–43

Berger, R. G. De Bont, J. A. M. Eggink, G. M. Da Fonseca, M. Gehrke, Gros, J-B. Keulen, F. V. Kings, U. Biotransformations in the Flavour Industry. In: SWIFT, K. A. D. Current Topics in Flavours and Fragrances. New York: Springer Netherlands (1999) 139-170.

Berger, R. G. Flavours and Fragrances: Chemistry, Bioprocessing and Sustainability. New York: Springer Berlin Heidelberg (2007) 648 p

Berger, R. G., Biotechnology of flavours—the next generation, Biotechnology Letters 11 (2009) 1651-1659

Beta, T. Duodu, K. G. Bioactive: antioxidants, Reference Module in Food Science (2016) doi:10.1016/B978-0-08-100596-5.00110-4

Bicas, J. K. Quadros, C. P. de. Néri-Numa, I. A. Pastore, G. M. Integrated process for co-production of alkaline lipase and R-(+)-α-terpineol by Fusarium oxysporum, Food Chemistry 120 (2) (2010) 452–456

Bicas, J.L Fontanille, P. Pastore, G. M. Larroche, C. Characterization of monoterpene biotransformation in two pseudomonads, Journal of Applied Microbiology 105 (6) (2008) 1991-2001.

Block, G. Patterson, B. Subar, A. 1992. Fruit, vegetables, and cancer prevention: a review of the epidemiological evidence, Nutrional Cancer 18 (1992) 1–29

Breitmeier, E. Terpenes: Importance, General Structure, and Biosynthesis. In: BREITMEIER, E. Terpenes: Flavors, Fragrances, Pharmaca, Pheromones. Berlin: Willey-VCH (2006) 1-9.

Burdock, G. A. Fenaroli's Handbook of Flavor Ingredients, 6 ed. Boca Raton: CRC Press (2010)

Calich, V. Vaz, C. Imunologia. 2º ed. Imunologia. Rio de Janeiro: Revinter, 2009, 323 p

Cartum, J. Neuroblastoma: o enigmático tumor da infância, Pediatria Moderna 48 (2012) 296-301

Carvalho, C. C. R. and Fonseca, M. M. R. Towards the bio-production of trans-carveol and carvone form limonene: induction after cell growth on limonen and toluene, Tetrahedron: Asymmetry, v. 14, n.5, 3925-3931 (2003)

Carvalho, C. C. R. de. Fonseca, M. M. R. da. Biotransformation of terpenes, Biotechnology Advances 24 (2) (2006) 134–142

Carvalho, C. C. R. de. Keulen, F. V. Fonseca, M. M. R. da. Biotransformation of Limonene-1,2-epoxide to Limonene-1,2-diol by Rhodococcus erythropolis Cells An Introductory Approach to Selective Hydrolysis and Product Separation, Food Technology and Biotechnology 38 (3) (2000) 181-185

Cathcart, M. K. Regulation of Superoxide Anion Production by NADPH Oxidase in Monocytes /Macrophages: Contributions to Atherosclerosis, Arteriosclerosis, Thrombosis, and Vascular Biology, 24 (2003), 23-28

Chapla, V. M. Estudo químico e biológico do fungo endofítico *Phomopsis* sp. isolado da Senna spectabilis. Dissertação (mestrado em Química). Universidade Estadual Paulista, Araraquara, 2010

Chen, H. modern solid state fermentation. New York: Springer (2013) 324 p

Chen, Z. Chu, H. Chyau, C. Chu, C. Duh, P. Protective effects of sweet orange (Citrus sinensis) peel and their bioactive compounds on oxidative stress, Food Chemistry 135 (2012) 2119–2127

Chi, D. S. Qui, M. Gu, Krishnaswamya. Li, C. Stone, W. Regulation of nitric oxide production from macrophages by lipopolysaccharide and catecholamines, Nitric Oxide 8 (2) (2003) 127–132

Cho, M. Hunt, T. K. Hussain, M. Z. Hydrogen peroxide stimulates macrophage vascular endothelial growth factor release, American Journal of Physiology - Heart and Circulatory Physiology 280 (2001) 2357-2363

Crizel, A. Jablonski, A.O. Rios, R. Rech, S.H. Flôres. Dietary fiber from orange byproducts as a potential fat replacer, Food Science and Technology 53 (2013) 9–14

Crowell, P. L. Elson. C. E. Bailey. H. H. Elegbede, A. Haag, J. D. Gould, M. N. Human metabolism of the experimental cancer therapeutic agent d-limonene, Cancer Chemotherapy and Pharmacology 35 (1994) 31-37

da Costa, J. P. Carvalhais, V. Amado, F. Silva, A. Nogueira-Ferreira, R. Ferreira, R. Helguero, L. Vitorino, R. Anti-tumoral activity of human salivary peptides, Peptides 71 (2015) 170-178

Danielski, L. Brunner, G. Schwänke, C. Zetzl, H. Hense, C. Donoso, J. P. M. Deterpenation of mandarin (Citrus reticulata) peel oils by means of countercurrent multistage extraction and adsorption/desorption with supercritical CO2, J. Supercritical Fluids 44 (2008) 215-324

Deepika, V.B. Murali, T.S. Satyamoorthy, K. Modulation of genetic clusters for synthesis of bioactive molecules in fungal endophytes: A review, Microbiological Research 182 (2016) 125–140

Demyttenaere, J. C. R. Kristof Van Belleghem, K. V. De Kimpe, N. Biotransformation of (R)-(+)- and (S)-(−)-limonene by fungi and the use of solid phase microextraction for screening, Phytochemistry 57 (2) (2001)199–208

Diaz, S. Espinosa, S. Brignole, E. A. Citrus peel oil deterpenation with supercritical fluids optimal process and solvent cycle design, J. Supercritical Fluids 35 (2005) 49-61

Duetz, W. A. Fjällman, A. H. M. Ren, S. Jourdat, C. Witholt, B. Biotransformation of D-Limonene to (+)trans-Carveol by Toluene-Grown Rhodococcus opacus PWD4 Cells, Applied and Environmental Microbiology 67 (6) (2001) 2829-2832

Ďuračková, Z. Free Radicals and Antioxidants for Non-Experts. In: LAHER, I. Systems Biology of Free Radicals and Antioxidants. Berlin: Springer Berlin Heidelberg (2014) 3-38

El-Akhal, F. Lalami, A. E. O. Guemmouh, R. Larvicidal activity of essential oils of Citrus sinensis and Citrus aurantium (Rutaceae) cultivated in Morocco against the malaria vector Anopheles labranchiae (Diptera: Culicidae), Asian Pacific Journal of Tropical Disease 5 (2015) 458-462

Ezeonu, F. C. Chidume, G. I. Udedi, S. C. Insecticidal properties of volatile extracts of orange peels, Bioresource Technology 76 (2001) 273-274

FDA. Limonene Glycol. Acesso em: 05/12/2015. Disponível em: http://www.accessdata.fda.gov/scripts/fcn/fcnNavigation.cfm?filter=1946-00-5&sortColumn=&rpt=eafusListing

Fiorentin, B.T. Menon, S.T.D. Barros, N.C. Pereira, O.C.M. Lima, A.N. Modenes Isotermas de sorção do resíduo agroindustrial bagaço de laranja Revista Brasileira de Engenharia Agrícola e Ambiental 14 (6) (2010) 653–659

Fischer, J. S. G. Mota e Silva, M. S. Paschoal, M. Carvalho, P. C. Carvalho, M. G. C. Efeito do álcool perílico na expressão gênica de células de adenocarcinoma de pulmão humano, Jornal Brasileiro de Pneumologia 31 (2005) 511-515

Fisher, K. Phillips, C. Potential antimicrobial uses of essential oils in food: is citrus the answer?, Trends in Food Science & Technology 9 (2008) 156-164.

Fouda, A. H. Hassan, S. E-D. Eid, A. M. Ewais, E. E-D. Biotechnological applications of fungal endophytes associated with medicinal plant Asclepias sinaica (Bioss.), Annals of Agricultural Sciences 60 (1) (2015) 95–104

Gad, S. C. Development of Therapeutic Agents Handbook. Wiley (2011) 680 p.

Gautam, N. Mantha, A. K. Mitall, S. Essential Oils and Their Constituents as Anticancer Agents: A Mechanistic View, BioMed Research International 2014 (2014) 23 p.

Gea-Bancloche, J. C. Immunomodulation. In: Runge, M. S. Patterson, C. Principles of Molecular Medicine. Nova Iorque: Springer Publishing, 2006. P 893-904

Gelb, M. Tamanol, F. Yokoyama, K. Esson, K. Gould, M. N. The inhibition of protein prenyltransferases by oxygenated metabolites of limonene and perillyl alcohol, Cancer Letters, 91 (1995) 169-175

Gosslau A. Chen, K. Y. Ho, C. Li, S. Anti-inflammatory effects of characterized orange peel extracts enriched with bioactive polymethoxyflavones, Food Science and Human Wellness 3 (2014) 26-35

Hassan, S. B. Gali-Muhtasib, H. Goransson, H. Larsson, R. Alpha Terpineol: A Potential Anticancer Agent which Acts through Suppressing NF-κB Signalling, Anticancer Researches 30 (2010) 1911-1919

Heath, H. B. Source Book of Flavors: AVI Sourcebook and Handbook Series. New York: Van Nostrand Reinhold (1981) 864 p

Hu, J. New Developments in terpenes research. Columbus: Nova Science Pub (2013) 294 p

Janeway, C.A. Travers, P. Walport T. M. Capra, J.D. Imunobiologia. O sistema imunológico na saúde e na doença. 5ª ed. Porto Alegre: ArtMed, 2002, 767 p

Jing, L. Zhang, Y. Fan, S. Gu, M. Guan, Y. Lu, X. Huang, C. Zhou, Z. Preventive and ameliorating effects of citrus D-limonene on dyslipidemia and hyperglycemia in mice with high-fat diet-induced obesity, European Journal of Pharmacology 715 (2013) 46–55

Kaur, J. Kaur, G. An insight into the role of citrus bioactives in modulation of colon cancer, Journal of Functional Foods 13 (2015) 239-261

Kusari, S. Singh, S. Jayabaskaran, C. Biotechnological potential of plant-associated endophytic fungi: hope versus hype, Trends in Biotechnology 32 (6) (2014) 297-303

Lima, N. G. P. B., Sousa, D. P. de, Pimenta, F. C. F., Alvez, M. F. Souza, F. S. de, Macedo, R. O., Cardoso, R. B., Morais, L. C. S. L. de, Diniz, M. F. F. M., Almeida, R. N. de. Anxiolytic-like activity and GC–MS analysis of (R)-(+)-limonene fragrance, a natural compound found in foods and plants, Pharmacology, Biochemistry and Behavior 103 (2013) 450-454

Liu, X. Dong, M. Chen, X. Jiang, M. Lu, X. Yan, G. Antioxidant activity and phenolics of an endophytic Xylaria sp. from Ginkgo biloba, Food Chemistry 105 (2) (2007) 548-554

Lu, Y. Zhao, S. Zhang, J. Zhang, H. Xie, Z. Cai, G. Jiang, W. Effect of orange peel essential oil on oxidative stress in AOM animals, International Journal of Biological Macromolecules 50 (2012) 1144-1150

MacMicking J. Xie Q. W. Nathan C. Nitric oxide and macrophage function, Annual Review of Immunology 15 (1997) 323-350

Mahmood, A. U. Greenman, J. Scragg, A. H.. Orange and potato peel extracts: Analysis and use as Bacillus substrates for the production of extracellular enzymes in continuous culture, Enzyme and Microbial Technology 22 (1998)130-137

Mantzouridou, F. T. Paraskevopoulou, A. Lalou, S. Yeast flavour production by solid state fermentation of orange peel Waste, Biochemical Engineering Journal 101 (2015) 1–8

Maróstica Jr, M. R. Pastore, G. M. Production of R-(+)-α-Terpineol by the biotransformation of limonene from orange essential oil, using cassava waste water as médium. Food Chemistry. v. 101, n. 1, p. 345-350 (2007)

Maróstica Júnior, M. R. Pastore, G. M. Biotransformation of limonene: a review of the main metabolic pathways, Química Nova 30 (2) (2007) 382-387

Maróstica Júnior, M. R., Silva, T. A. A. R., Franchi, G. C., Nowill, A., Pastore, G. M., Hyslop, S. Antioxidant potential of aroma compounds obtained by limonene biotransformation of orange essential oil, Food Chemistry 116 (2009) 8-12

Miller, J. A. Thompson, P. A. Hakim, I. A. Chow, H. S. Thomson, C. A. D-Limonene: a bioactive food component from citrus and evidence for a potential role in breast cancer prevention and treatment, Oncology Review 5 (2011) 31-42

Molina, G. Bution, M. L. Bicas, J. L. Dolder, M. A. H. Pastore, G. M. Comparative study of the bioconversion process using R-(+)- and S-(–)-limonene as substrates for Fusarium oxysporum 152B, Food Chemistry 174 (2015) 606–613

Morath, S. U. Hung, R. Bennet, J. W. Fungal volatile organic compounds: A review with emphasis on their biotechnological potential, fungal biology reviews 26 (2012) 73-83

Morcia, C. Tumino, G. Ghizzoni, R. Terzi, V. Carvone (Mentha spicata L.) Oils. In: PREEDY, V. Boston: Elsevier Essential Oils in Food Preservation, Flavor and Safety (2015) 309-316

Mordor Intelligence LLP, Global Flavor and Fragrance Industry 2016 Market Research Report. Disponível em: http://www.reportlinker.com/p03024563/Brazil-Food-Flavor-and-Enhancer-market-Growth-Trends-Forecast-for-the-period.html

Mousa, W. K. Raizada, M. N. The Diversity of Anti-Microbial Secondary Metabolites Produced by Fungal Endophytes: An Interdisciplinary Perspective, Frontiers in Microbiology 4 (2013) 1-18

Mueller, G.M. Bills, G.F. Foster, M.S. Biodiversity of Fungi: Inventory and Monitoring Methods. Boston: Elsevier Academic Press (2004) 777 p

Mukherjee, B. B. Kraidman, G. Hill, I. D. Synthesis of Glycols by Microbial Transformation of Some Monocyclic Terpenes, Applied Microbiology 25 (3) (1973) 447-453

Murray, M. T. The Complete Book of Juicing, Revised and Updated: Your Delicious Guide to Youthful Vitality. Clarkson Potter (2013)

Murthy, K. N. C., Jayaprakasha, G. K., Patil, B. S. D-limonene rich volatile oil from blood oranges inhibits angiogenesis, metastasis and cell death in human colon cancer cells, Life Sciences 91 (2012) 429-439

Nakatani, N.. Phenolic antioxidants from herbs and spices, Biofactors 13 (2000) 141–146

Netto, B. Press Release: Análise de Dados de Estimativa de Safra 2015/16. Araraquara: CitrusBR – Assossiação nacional dos exportadores de sucos cítricos, 2015

Nicolini, L. Hunolstein, C. V. Carilli, A. Solid state fermentation of orange peel and grape stalks by Pleurotus ostreatus, Agrocybe aegerita, and Armillariella mellea, Applied Microbiology and Biotechnology 26 (1) (1987) 95-98

NPCS Board of Consultants & Engineers. The Complete Technology Book on Alcoholic and Non-Alcoholic Beverages (Fruit Juices, Whisky, Beer, Rum and Wine). Delhi: Asia Pacific Business Press Inc. (2008) 824 p

Olano, C. Méndez, C. Salas, J. A. Strategies for the Design and Discovery of Novel Antibiotics using Genetic Engineering and Genome Mining. In: VILLA, T. G. VEIGA-CRESPO, P. Antimicrobial Compounds. Berlin: Springer Berlin Heidelberg (2013) 1-25

Oses, R. Valenzuela, S. Freer, J. Baeza, J. Rodríguez, J. Evaluation of fungal endophytes for lignocellulolytic enzyme production and wood biodegradation, International Biodeterioration & Biodegradation 57 (2) (2006) 129-135

Pourbafrani, M. Forgács, G. Horváth, I. S. Niklasson, C. Taherzadeh M. J. Production of biofuels, limonene and pectin from citrus wastes Bioresource Technology 101 (11) (2010) 4246-4250

Poon, G. K. Vigushin, D. Griggs, L. J. Rowlands, M. G. Coombes, R. C. Jarman, M. Identification and characterization of limonene metabolites in patients with advanced cancer by liquid chromatography/mass spectrometry, Drug Metabolism & Disposition 24 (5) (1996) 565-571

Rakshith, D. Santosh, P. Satisha, S. Isolation and characterization of antimicrobial metabolite producing endophytic *Phomopsis* sp. from Ficus pumila Linn. (Moraceae), International Journal of Chemical and Analytical Science 4 (3) (2013) 156–160

Reineccius, G. Sourcebook of Flavors 2nd edition. New York: Springer (1998) 928 p

Rezzadori, K. Beneditti, S. Amante, E. R. Proposals for the residues recovery: orange waste as raw material for new products. Food Bioprod Process 90 (4) (2012) 606-614

Roberto, D. Micucci, P. Sebastian, T. Graciela, F. Anesini, C. Antioxidant Activity of Limonene on Normal Murine Lymphocytes: Relation to H2O2 Modulation and Cell Proliferation, Basic & Clinical Pharmacology & Toxicology 106 (2009) 38-44

Rodriguez, R. J. White Jr, J. F. Arnold, A. E. Redman, R. S. Fungal endophytes: diversity and functional roles, New Phytologist 182 (2) (2009) 314-330

Rottava, I. Cortina, P.F. Martello, E. Cansian, R. L. Toniazzo, G. Antunes, O.A. Oestreicher, E. G. Treichel, H. de Oliveira, D. Optimization of α-Terpineol production by the biotransformation of R-(+)-limonene and (-)-β-pinene, Applied Biochemistry and Biotechnology 164 (4) (2011) 514-523

Rubel, R. Santa, H. D. S. Fernandes, L. C. Bonatto, S. J. R. Bello, S. Figueiredo, B. C. Lima Filho, J. H. Santos, C. A. M. Soccol, C. R. Hypolipidemic and antioxidant properties of Ganoderma lucidum (Leyss:Fr) Karst used as a dietary supplement, World Journal of Microbiology and Biotechnology 27 (2011) 1083-1089

Rubel, R. Santa, H. S. D. Bonatto, S. J. R. Bello, S. Fernandes, L. C. Bernardi, R. D. Gern, J. Santos, C. A. Soccol, C. R. Medicinal Mushroom Ganoderma lucidum (Leyss: Fr) Karst. Triggers Immunomodulatory Effects and Reduces Nitric Oxide Synthesis in Mice, Journal of Medicinal Food 13 (2010) 142-148

Rueda, A. Q. Rodriguez, I. G. Arantes, E. C. Setúbal, S. S. Calderon, L. A. Zuliani, J. P. Stábeli, R. G. Soares, A. M. Biochemical Characterization, Action on Macrophages, and Superoxide Anion Production of Four Basic Phospholipases A2 from Panamanian Bothrops asper Snake Venom, Biomedical Research International 2013 (2013) 9p

Rukachaisirikul, V. Sommart, U. Phongpaichit, S. Sakayaroj, J. Kirtikara, K. Metabolites from the endophytic fungus *Phomopsis* sp. PSU-D15, Phytochemistry 69 (3) 783-787, 2008.

Santi, G. Crognale, S. D'Annibale, A. Petruccioli, M. Ruzzi, M. Valentini, R. Moresi M. Orange peel pretreatment in a novel lab-scale direct steam-injection apparatus for ethanol production, Biomass and Bioenergy 61 (2014) 146–156

Schepetkin, I A.; Quinn, M. T. Botanical polysaccharides: macrophage immunomodulation and therapeutic potential, International immunopharmacology, 6 (2006) 317-333

Serra, S. Recent Developments in the Synthesis of the Flavors and Fragrances of Terpenoid Origin. In: RAHMAN, A-U. Studies in Natural Products Chemistry. Boston: Elsevier (2015) v. 46, 201-226

Shamtsyan, M. Konusova, V. Maksimova, Y. Goloshchev, A. Panchenko, A. Simbirtsev, A. Petrischev, N. Denisova, N. Immunomodulating and anti-tumor action of extracts of several mushrooms, Journal of biotechnology 113 (2004) 77-83

Shrader, J. Microbial Flavour Production. In: BERGER, R. G. Flavours and Fragrances: Chemistry, Bioprocessing and Sustainability. New York: Springer Berlin Heidelberg (2007) 507-574

Silva, W.D. Mota, I. Bier imunologia básica e aplicada. 5.ed. Rio de Janeiro: Guanabara Koogan, 2003, 388 p

Simon, J. A. Szankasi, P. Nguyen, D. K. Ludlow, C. Dunstan, H. M. Roberts, C. J. Jensen, E. L. Hartwell, L. H. Friend, S. H. Differential toxicities of anticancer agents among DNA repair and checkpoint mutants of Saccharomyces cerevisiae, Cancer Research 60 (2) (2000) 328-333

Singh, P. Shukla, R. Prakash, B. Kumar A. Singh, S. Mishra, P. K. Dubey, N. K. Chemical profile, antifungal, antiaflatoxigenic and antioxidant activity of Citrus maxima Burm. and Citrus sinensis (L.) Osbeck essential oils and their cyclic monoterpene, DL-limonene, Food and Chemical Toxicology 48 (2010) 1734-1740

Singhania, R. R. Patel, A. K. Soccol, C. Pandey, A. Recent advances in solid-state fermentation, Biochemical Engineering Journal 44 (1) (2008) 13-18

Soccol, C. R. Medeiros, A. B. P. Vanderberghe, L. P. S. Soares, M. Pandey, A. Production of Aroma Compounds. In: PANDEY, A. SOCCOL, C. R. LARROCHE, C. Current Developments in Solid-state Fermentation. New York: Springer (2008) 356-376

Steinbrecher, R. Hauff K. Riissler, J. Diirr, M. Seufert, G. Monoterpene Emission from Valencian Citrus Belt, Spain Soils in Orange Plantations of the Spain. Physics and Chemistry of the Earth B. 24 (1999) 695-698

Strobel, D. Daisy, B. Bioprospecting for Microbial Endophytes and Their Natural Products, Microbiology and Molecular Biology Reviews 67 (4) (2003) 491-502

Subramaniyam, R; Vimala, R. Solid state and submerged fermentation for the production of bioactive substances: a comparative study, International Journal of Science and Nature 3 (2012) 480-486

Sun, X. Guo, L-D. Endophytic fungal diversity: review of traditional and molecular techniques, Mycology: An International Journal on Fungal Biology 3 (1) (2012) 65-76

Tai, Y-N. Xu, M. Ren, J-N. Dong, M. Yang, Z-Y. Fan, G. Optimisation of α-terpineol production by limonene biotransformation using Penicillium digitatum DSM 62840, Journal of Science and Food Agriculture (2015) 8 p DOI 10.1002

Tan, Q. Day, D. F. Cadwallader, K. R. Bioconversion of (R)-(+)-limonene by P. digitatum (NRRL 1202). Process Biochemistry. 33 (1) (1998) 29-31

Tang, D. Shi, Y. Kang, R. Li, T. Xiao, W. Wang, H. Xiao, X. Hydrogen peroxide stimulates macrophages and monocytes to actively release HMGB1, Journal of Leukocyte Biology, 81 (2007) 741-747

Trytek, M. Fiedurek, J. Biotransformation of d-limonene to carvone by means of glucose oxidase and peroxidase, Acta Biochimica Polonica 51 (1) (2002) 57-62

Udayanga, D. Liu, X. McKenzie, E. H. C. Chukeatirote, E. Ali H. A. Bahkali, A. H. A. Hyde, K. D. The genus *Phomopsis*: biology, applications, species concepts and names of common phytopathogens, Fungal Diversity 50 (1) (2011) 198-225

United States Department of Agriculture. Citrus: World Markets and Trade. 2016. Available in: <https://apps.fas.usda.gov/psdonline/circulars/citrus.pdf>, Accessed in: 23/09/2016.

Vanek, T. Valterová, I. Vanková, R. Vaisar, T. Biotransformation of (+)-limonene using Solanum aviculare and Dioscorea deltoidea immobilized plant cells, Biotechnology Letters, v. 21, p;625-628 (1999)

Velázquez-Nuñez, M. J. Avila-Sosa, R. Palou, E. López-Malo, A. Antifungal activity of orange (Citrus sinensis var. Valencia) peel essential oil applied by direct addition or vapor contact, Food Control 31 (2013) 1-4

Venugopalan, A. Srivastava. S. Endophytes as in vitro production platforms of high value plant secondary metabolites, Biotechnology Advances 33 (6) (2015 873–887

Viniegra-Gonzàlez, G. Solid state fermentation: Definition, Characteristics, Limitations and Monitoring. In: ROUSSOS, S. LONSANE, B. K. RAIMBAULT, M. VINIEGRA-GONZÀLEZ, G. Advances in Solid State Fermentation. New York: Springer Netherlands (1997) 5-22

Wong, W. S. McLean, A. E. Effects of phenolic antioxidants and flavonoids on DNA synthesis in rat liver, spleen, and testis in vitro, Toxicology 139 (1999) 243–253

Woo, C. W. Man, R. Y. Siow, Y. L. Choy, P. C. Wan, E. W. Lau, C. S. Ganoderma lucidum inhibits inducible nitric oxide synthase expression in macrophages, Molecular and Cellular Biochemistry 275 (2005) 165-171

Yadava, A. R. Chauhan, A. S. Rekha, . M. N. Ramteke, R. S. Flavour quality of dehydrated lime [Citrus aurantifolia (Christm.) Swingle], Food Chemistry 85 (2004) 59-62

Yang, F-C. Ma, T-W. Lee, Y-H. Reuse of citrus peel to enhance the formation of bioactive metabolite-triterpenoid in solid-state fermentation of A. cinnamomea, Biochemical Engineering Journal 78 (2013) 59–66

Capítulo 2 - Liquefied gas extraction: A new method for the recovery of terpenoids from agroindustrial and forest wastes

Artigo publicado no Journal of Supercritical Fluids 110(2016) 97-102

ABSTRACT

Terpenoids are important ingredients in perfumery, and terpene residues have shown potential as new sources of these compounds. This study aimed to apply gas extraction with liquefied petroleum gas (butane/propane) as a clean technology to extract terpenes from agroindustrial and forest wastes. The extracts obtained were characterized by gas chromatography–mass spectrometry (GC-MS), and compared to extracts obtained using traditional organic solvent extraction (hexane and dicholoromethane:n-pentane). The oil extraction yields were 5.36% for the orange waste, 2.1% for the apple pomace, 2.32% for the pine needle and 0.6% for the pine wood shavings. The extract obtained using this new method on orange waste contained the highest concentration of limonene (95.32 g/100 g of extract), α-pinene (0.40) and β-pinene (0.23) among all of the solvents as well as the largest extracted amount (0.051 g/g of dry substrate). Based on the GC-MS analyses, the LPG extraction displayed less decomposition or modification of the compounds.

Keywords:

Terpenoids, orange waste, apple pomace, Pinus, Pressurized LPG, extraction

1. Introduction

Solvent extraction has been widely used for the separation, purification, and recovery of substances due to the simplicity of its equipment and operation. However, the disadvantages of traditional solvent extraction have been recently recognized. The main disadvantages of conventional Soxhlet extraction include (1) a long extraction time, (2) the use of a large amount of solvent, (3) an inability to accelerate the process using agitation, (4) an evaporation or concentration process being necessary due to the high quantity of solvent used in the process and (5) the possibility of thermal degradation of the targeted compounds occurring during extractions over a long period of time at the

solvent boiling point [1]. Most of these disadvantages are applicable to other extraction methods, such as the amount of solvent used. Dichloromethane:n-pentane (DMP) extraction is fast and simple but the low boiling point of the solvents can be a problem during the analysis even though it makes the sample easier to concentrate after the extraction. Thermal degradation is a major issue for the extraction of terpenoids. α-Pinene, limonene, camphor, citronellol, carvacrol, camphene, Δ^3-carene, and r-terpinene have high degradation indices at temperatures above 100ºC, under subcritical water conditions [2] and in the presence of hot air [3]. Due to their limited water solubility, terpenoids are commonly extracted on a large scale with methanol or 2-propanol, ethyl acetate and light petroleum (1:1:1) at temperatures that range from 40ºC to 190ºC [4].

The use of supercritical fluids is an alternative that replaces or supplements traditional industrial methods similar to using solvent extraction and pressing in oil extraction. This technique has several advantages over conventional methods due to the absence of retained solvent in the extracts. These advantages include low temperature extraction, elevated product quality and decreased energy usage [5]. Despite the method using liquefied petroleum gas (LPG) not under supercritical fluid conditions, the advantages are the same as those described for this method due to its general similarities. In addition, the extractor designed to pressurized LPG extraction is suitable for use in a fast, clean and cheap method for the extraction of terpenes and their derivatives. Pressurized LPG extraction has been rarely studied but has been previously employed for steroid extraction from fruits of the pepper family [6] and to enhance the enzymatic hydrolysis of sugarcane bagasse [7]. The current method does not exhibit the main recurring problem in terpene extraction and offers a series of advantages regarding ecological and technical issues. Therefore, this method offers a solution that can be applied for the recovery of terpenes in industrial processes and on the laboratory scale.

Currently, environmental pollution has become an important issue due to increasing global ecological concerns. New substrates for extraction, trade and use of byproducts as substrates in processes are an emerging issue not only due to environmental concerns but also for financial reasons. Therefore, the determination of the profiles of volatile waste enables several options including seeking grants to create new technologies that employ these promising substrates.

Citrus fruits have high concentrations of limonene [8-11]. Therefore, its wastes may be of great interest to various areas that can take advantage of this terpene. The processing of orange juice is one of the most important industries in the world, producing an enormous amount of process residues. This residue constitutes approximately 50% of the weight of the fruit and provides excellent models for value-added products. According to Mahmood [12], bagasse orange, bark and seeds comprises approximately 46% of the fruit with the juice making of 54% of the fruit.

Different conifer species have high concentrations of terpenes [13-14]. An industrial waste rich in α-pinene and β-pinene is produced by paper industries, and pine needles (Pinus leaf), Pinus bark and sawdust from the timber industry are also natural sources of these terpenes. α-Pinene and β-pinene are monoterpenes that are widely distributed in nature as well as in industrial wastes with minimal commercial value [15]. Pinus bark represents approximately 10 to 15% of the total weight of the tree [16]. The discarded Pinus bark from production processes is still an attractive and important renewable source of biomass. However, this material has a high content of polyphenols and low carbohydrate concentration [17].

The main byproduct of apple agribusiness (apple bagasse) can represent 20-40% of the total apple quantity processed and is currently being used as animal feed or organic fertilizer [18]. Some terpenes are found on apples (i.e., limonene) [19-20], and other terpenes, such as α-pinene, β-pinene and linalool, have been reported [21].

Other terpenes are present in many plant species and part of the main composition of many essential oils. Therefore, orange waste, apple pomace and pine substrates, terpenes, such as camphene, myrcene, p-cymene, terpinolene, cadinene, sabinene, longifolene, germacrene, linalool, terpinene, β-phellandrene, 3-carene, spathunelol, α-farnesene and citral have been found [8-10, 13-15, 19-21]. In addition to those previously mentioned, many of these terpenes have important biological properties and can be used as precursors to products of interest in various industrial fields. Despite the fact that the agro-industrial wastes involved in this study predominantly contain limonene, α-pinene and β-pinene, a large number of other terpenes were detected at lower concentrations.

The main objective of this study was to apply pressurized liquefied petroleum gas extraction (butane/propane) as an alternative to the use of traditional solvents for the recovery of terpenoids from agroindustrial and forest wastes and confirm the use of these wastes as a source of terpenoids.

2. Material and methods

2.1 Residues

The residues from the extraction of orange juice (peel and pulp) were obtained from a canteen located at the Federal University of Paraná (Curitiba, PR, Brazil). Pinus needles and bark were collected at the forestry area of Arauco Forest of Brazil (Campo do Tenente, PR, Brazil). The pinus samples collected belong to the species Pinus taeda. Pine wood shavings were acquired from Aliança Ltda. (Curitiba, PR, Brazil). The collected pinus belongs to the species Pinus ellioti. These shavings are residues that are larger in size than that of sawdust particles resulting from cutting wood. The apple pomace used in this study was donated by Agrícola Fraiburgo S.A. (Videira, SC, Brazil) and consisted of a mixture of Fuji and Gala apples with a minor component consisting of Granny Smith, Pink Lady, Colorida, Imperial, Catarina, Joaquina and Eva apples. All of the materials were dried in an oven with circulating air at 60ºC, milled in a Willey-type knife mill (De Leo Equipamentos para Laboratório Ltda) and classified granulometrically below 0.8 mm.

2.2 LPG extraction

The extraction was performed in a portable extractor for use with liquefied gases developed by Oliveira [22]. Liquefied petroleum gas (LPG), which consists of a mixture of butane and propane, was used as the extractor solvent. The used gas consisted of propane 25% ± 5% and iso-butane + n-butane 75% ± 5% w/w. The liquid-phase solvent was transferred into the equipment vessel where it came into contact with 5 g of dried material. The equipment extraction flowchart is shown in Figure 1. The extraction was performed using the liquefied form of butane/propane. The vapor pressure of the LPG was estimated to be 544.93 ± 43.4 kPa .

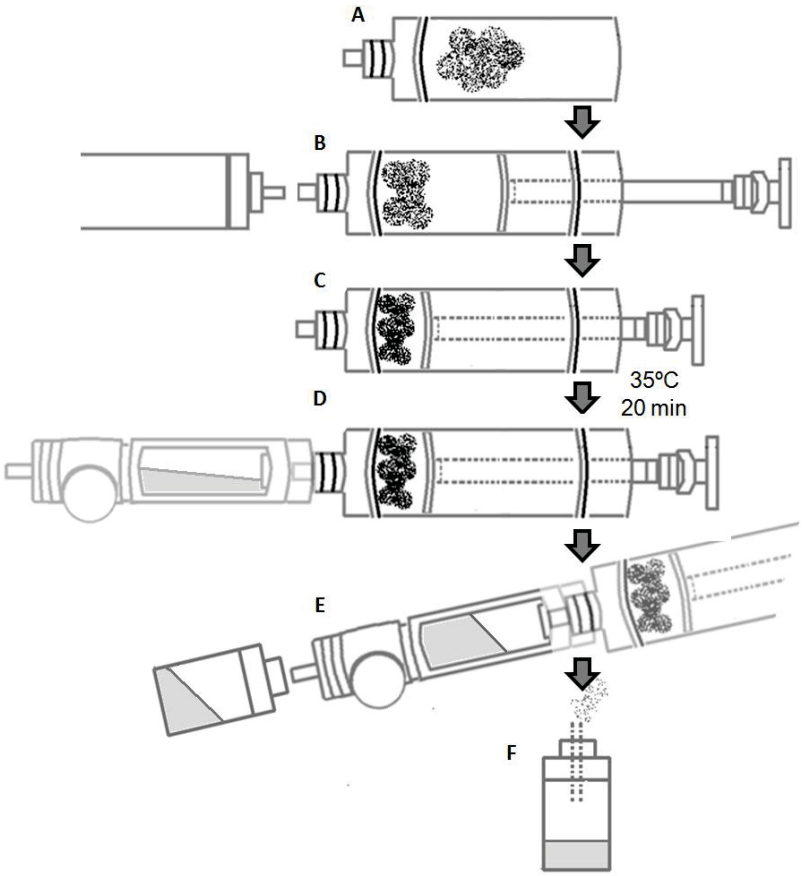


Fig. 1 LPG extractor flow chart: A: Sample Filling. B: Coupling of the container and LPG injection. C: Plunger compression. D: Recovery compartment coupling and extract recuperation. E: Extract transfer to a vial. F: Solvent flash evaporation.

Each cycle of extraction used 15 g of LPG (Volcano Isqueiros Ltda, São Paulo, SP, Brazil). The exposure time of the sample material was set to 20 minutes per cycle at 35ºC in an oven. The required number of cycles was previously determined according to efficiency assays where five cycles was consider to be 100%. The extract was transferred to a sealed vial without contact with the environment using a needle attached to the extractor. Then, the LPG was evaporated under atmospheric pressure. All of the assays were performed in triplicate for each of the extraction methods.

2.3 Traditional method

The sample extraction with n-hexane (>95%) was performed in a Soxhlet apparatus according to the analytical standards of Instituto Adolfo Lutz [23] as well as Silva et al. [24] and Costa et al. [25] who used hexane as a solvent to obtain the best terpene profile. The extraction required at least 8 cycles, and an overall time of 6 hours.

The extraction with dichloromethane and n-pentane was adapted from a protocol reported by Shashirekha et al. [21]. Using 150 mL of a 1:1 solution consisting of dichloromethane and n-pentane, 5 g of substrate were subjected to extraction three times at room temperature. This solution was previously maintained overnight at -20°C. Then, anhydrous sodium sulfate was used to remove the moisture from the samples. The samples were concentrated in a Vigreux column at a temperature of less than 35°C.

2.4 Volatile compounds analysis

The volatile compounds present in the extracts were analyzed according to the method previously reported by Demyttenaere, Vanorschelde and Kimpe [26]. The equipment involved a GC-17A gas chromatograph from Shimadzu with a flame ionization detector and a HP-5 column (30 m x 0.32 mm) with nitrogen as the carrier gas. The injector temperature was 250°C, and the detector temperature was 280°C. In addition, a temperature program was employed where the oven was initially at 40°C for 2 min and increased at 5 °C/min to 150°C. From 150ºC to 170º C, the rate of increase was 10 ºC/min, and then, an increase of 30ºC/min was employed until a final temperature of 250°C was reached. This temperature was maintained for 2 min. A split ratio of 1:40 was used. The extracts were diluted in n-hexane (>95%). The results are expressed as % peak area of the product compared to the peak area of limonene.

The identification of the volatile compounds was performed using a gas chromatograph coupled with a mass detector (GC-MS) (Varian CP 3800/Saturn Model 2000) equipped with a column CP-Sil 8 CB (30 m x 0.25 mm). The initial temperature was 60 ºC with an increase of 3 °C/min to 250°C. The split was 1:200. The scan range was 30 to 500 m/z. The identification of the extract components was evaluated by comparison with the MS standards of the National Institute of Standard and Technology [27] in addition to comparison of the arithmetic index of the compounds to the specific literature [28]. All of the analyses were performed for each of the triplicates.

3. Results and Discussion

In the absence of a standard methodology for the analysis of terpenes, Soxhlet (n-hexane), dichloromethane:n-pentane (DMP) and the equipment developed for LPG extraction were employed to test their efficiency for the extraction of terpenes. Soxhlet

was selected because it is commonly used as a standard method for comparison [24, 27, 29, 30], and DMP extraction was selected as an alternative extraction method that has been reported in the literature [21]. The main goal of this step was to quantitatively determine the method with the highest extraction yield. The extraction yield was calculated as the ratio of the mass of extract obtained and the mass of dry material used for extraction (Figure 2). Based on the differences in the amount of extract from each method in Figure 2, LPG was the best for the orange waste (5.36 g/100 g), Soxhlet was the best for pine needle (4.13 g/100 g) and apple pomace (3.22 g/100 g). In addition, for the pine wood shavings (1.54 g/100 g), similar results were obtained for the Soxhlet method and DMP extraction. This difference may due to various factors, such as the composition of the extracts and solvent affinity. It is important to note that LPG exhibited the highest yield for orange waste extraction because among the studied substrates, it is the richest in terpene and the best reference for the terpene model.

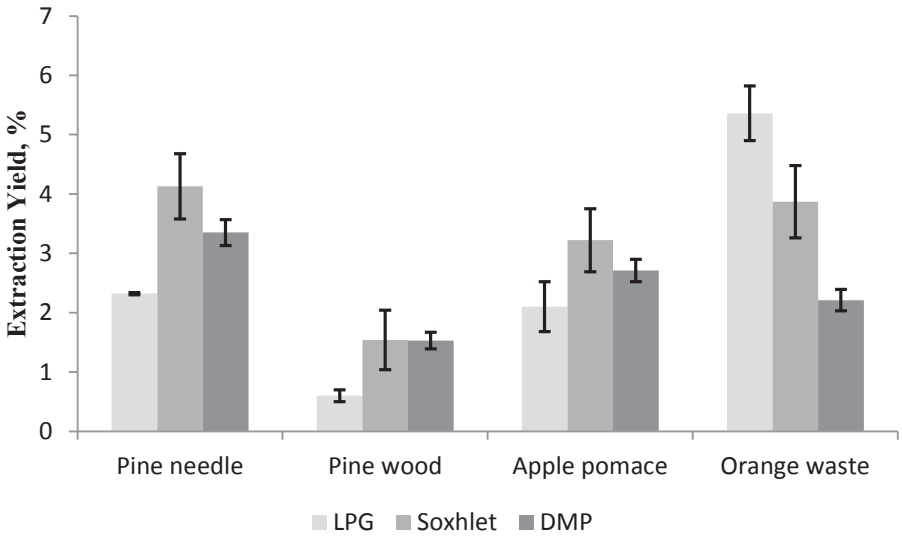


Fig. 2 Extraction yield obtained from pine needles, pine wood shavings, apple pomace and orange waste using different extraction methods.

Table 1 shows the extract composition for the main terpenes (i.e., limonene, β-pinene and α-pinene) present in the extract from the materials. The studied substrates have been previously investigated to determine the concentrations of terpenes. However, it is important to note that the substrates studied are agroindustrial and forest wastes, and studies of the volatile composition of residues are rare in the literature. The apple

pomace is expected to contain a small amount of monoterpenes. Limonene was found as the only terpene in Pink Lady apples but only at low concentrations [19], which is similar to this study where the limonene was the main monoterpene (3.42 g/100 g of extract) α-Pinene (0.64 g/100 g) and β-pinene (2.28 g/100 g) were also found at lower concentrations. Limonene was also to be the only terpene in Mondial Gala apple by Echeverría et al. [20] Shashirekha et al. [21] found α-pinene, β-pinene, linalool, germacrene-D, and spathulenol in Annona squamosa apples.

Table 1: Primary terpenic composition of the extracts obtained by the different extraction methods expressed in g/100 g.

Sample	Method	R-(+)-Limonene	β-pinene	α-pinene
Pine needle	DMP	0.87 ± 0.23	13.83 ± 3.83	1.08 ± 0.34
	LPG	0.48 ± 0.17	7.37 ± 3.07	0.57 ± 0.19
	Soxhlet (hexane)	1.25 ± 0.22	3.76 ± 1.31	0.70 ± 0.07
Pine Wood Shavings	DMP	0.70 ± 0.15	8.43 ± 1.81	2.99 ± 0.59
	LPG	0.38 ± 0.092	6.34 ± 1.24	2.93 ± 0.63
	Soxhlet (hexane)	2.414 ± 0.64	3.85 ± 0.82	0.79 ± 0.14
Apple pomace	DMP	Nd*	Nd*	Nd*
	LPG	0.50 ± 0.18	0.52 ± 0.12	Nd*
	Soxhlet (hexane)	3.42 ± 0.55	2.28 ± 0.84	0.64 ± 0.24
Orange waste	DMP	94.75 ± 0.44	0.29 ± 0.08	0.19 ± 0.06
	LPG	95.32 ± 0.94	0.4 ± 0.04	0.23 ± 0.005
	Soxhlet (hexane)	84.45 ± 0.08	0.31± 0.103	0.14 ± 0.04

*Not detected

For the pine residues, very low values were found. The maximum amount of β-pinene was 13.83 g/100 g in the extract of pine needles (Pinus taeda), and 2.99 g/100 g of α-pinene was found in the extract of wood shavings (Pinus ellioti). High values for α-pinene and β-pinene, which are the most distinctive components in essential oil, are typically reported. Krassimir et al. [31] reported as much as 62.01% α-pinene, 10.56% β-pinene and 3.57% limonene in the essential oil of Pinus. Semiz et al. [14] found 43.7% to 89% α-pinene, 1.2 to 10.3% β-pinene and 1.3 to 4.9% limonene. Barnola et al. [32] found lower values in Pinus caribae with 8.25% α-pinene, 2.31% β-pinene and 5.64% limonene. Most of the values reported for α-pinene in the literature are quite high and indicate that monoterpene is the main constituent in essential oils. The low concentration of α-pinene and β-pinene in the waste of two different species of Pinus

may be due to the results for the essential oil of Pinus being obtained from fresh samples and not from agricultural residues from forest soil.

The orange waste contained 5.36 g/100 g of essential oil in its composition and a very significant composition of limonene (95.32 g/100 g of extract) from extraction with butane gas (LPG extraction), which released much more essential oil than that obtained by Soxhlet extraction (3.88) and DMP (2.21). For the essential oil of oranges, the reported values for limonene are quite high. In particular, Danielski et al. [33] reported a value of 95.95%.

3.1 Comparison of solvents for terpene extraction

The major differences observed between the different extraction solvents were due to the quantitative amount of compounds in the extracts. In addition, minor changes were observed in the qualitative composition of the extracts.

It was applied extraction assays to orange waste to confirm the number of cycles (Figure 3). The highest concentration of terpenes in orange waste makes it the most accurate substrate. The results in Figure 3 indicate that the first cycle represents a very efficient cycle and the second cycle was also very efficient. The efficiency of the third, fourth and fifth extractions decreased. Three cycles of the LPG method resulted in an additional 5.36% extraction yield from the orange waste oil, and with four extractions, this yield reached 6.56%. In comparison to other methods, LPG is advantageous due to the small number of cycles (i.e., two), which resulted in 83.1% extraction efficiency for the orange waste compared to that with five cycles. Due to the low efficiency of the third cycle (5-7%), the limitations of time and large amount of sample, only two cycles were employed in further studies. A quantitative analysis was performed, which confirmed that the composition did not change among the cycles. Soxhlet extraction is not controlled by the number of cycles but by the extraction time. However, the hexane extraction required an average of eight Soxhlet cycles per sample. DMP requires three cycles, and LPG extraction required only two cycles (83.10% ± 0.15).

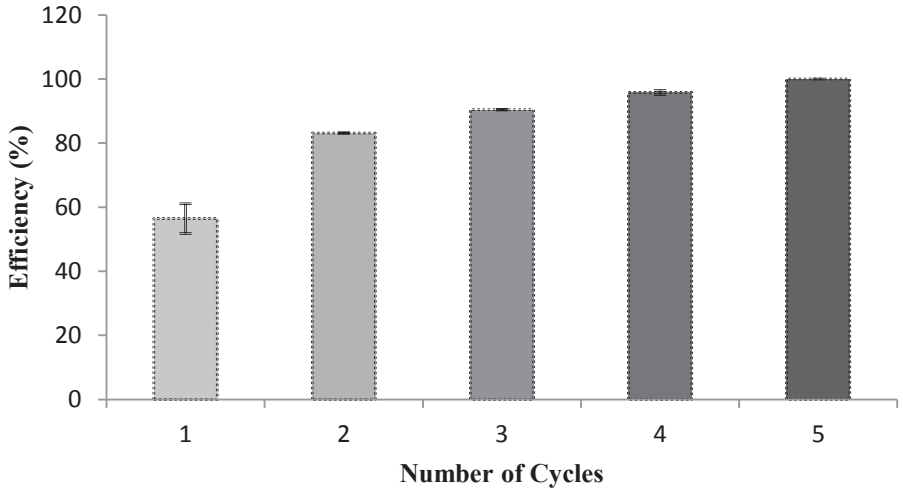


Fig. 3 Cumulative eEfficiency of LPG extraction of orange residues

Small quantity of terpenes was found in the extracts of apple pomace and pine residues, not only after the extractions with Soxhlet but also with dichloromethane/ n-pentane. Other compounds that were not of interest were found in these extracts. The LPG method was effective for extracting the essential oil of orange waste (5.36 g/100 g) with high concentrations of limonene (95.32 g/100 g of extract). In addition, the butane/propane in the LPG method may easily extract this limonene, resulting in a higher amount of extract. Due to this high affinity for terpenes, non-terpenoid compounds were not detected by GC-MS, which is an advantage of the LPG method compared to the other methods (Table 2). This method does not employ heating, which makes it more stable and prevents changes in the composition of the samples. Gomes et al. [34] reported the degradation of geraniol into linalool due to a long period of heating and exposure to water vapor during extraction. Yang et al. [2] and Mcgraw et al. [3] also observed the degradation of terpenes at high temperatures. The existence of different compounds, such as linalool, myrcene and butylated hydroxytoluene, from the LPG method compared to that from other solvents is exclusive to the LPG method. In some methods, 2,2-dimethyl-1-pentane and trans-sabinene hydrate were detected, which were not observed using this method. These substances could have been formed by the modification of other compounds due to heating.

Table 2: Compounds identified by GC-MS analysis of apple pomace and orange waste extracts under the different extraction methods.

Compounds/ Method	Apple pomace			Orange waste			Experimental AI	Literature AI
	1	2	3	1	2	3	-	-
Dimethyl Sulfoxide		x					832	Nl*
2,2-dimethylpentanol		x					886	Nl*
α-pinene	x			x	x	x	934	932
β-pinene	x		x	x	x	x	973	974
Myrcene				x	x	x	988	988
Limonene	x	x	x	x	x	x	1029	1024
Linalool						x	1100	1095
Trans-sabinene hydrate				x	x	x	1101	1098
α-Terpineol				x	x	x	1190	1186
Trans-dihydrocarvone				x	x	x	1201	1200

(1) Soxhlet (hexane); (2) DMP; (3) LPG.

*Nl: The arithmetic index was calculated, but the specific literature for comparison is not available for these compounds.

A qualitative comparison of the composition of the apple pomace and orange waste in relationship to the extraction method is shown in Table 2. The arithmetic indices (AIs) of all of the compounds are very similar to the ones verified in the literature [28] for the chromatography method used. According to previous studies [11, 33] α-pinene, β-pinene, myrcene, limonene, terpinolene, α-terpineol, linalool, citral, sabinene and terpinolene are some of the terpenoids commonly found in the essential oil of citrus fruits. As noted by several authors [13-15], α-pinene, β-pinene, caryophyllene, myrcene, limonene, α-copaene, α-terpineol, α-humulene, β-elemene, β-phellandrene and γ-cadinene are some of the major terpenes found in the essential oil of the Pinus samples, and these terpenes have also been identified in pinus needles and shavings by GC-MS (data not shown).

For most of the samples, the LPG method produced a high diversity of compounds, and for the apple pomace, the dichloromethane/n-pentane method was more efficient, which is most likely due to the higher polarity of the obtained compounds. Based on this characterization, compounds, such as linalool and trans-dihydrocarvone, were found in orange waste.

4. Conclusions

Three different extraction methods were applied to four different agroindustrial wastes to analyze their terpene volatile composition. Among the pine needles and pine wood shavings, apple pomace and orange waste, different extraction methodologies and solvents were determined to be more suitable for each substrate. For apple pomace, the LPG method provided lower concentrations of terpenoids compared to that from the Soxhlet method, and the DMP extraction was not able to detect the three major terpenoids (i.e., limonene, α-pinene and β-pinene). The LPG method produced the lowest extraction yield for the pine wood shavings but good results for the oil composition. For the orange waste, the LPG method produced the best result among the studied methods. This method had the highest yield (5.36 g/100 g) and was superior for the limonene concentration (0.051 g/g of dry substrate).

The orange waste was the substrate with the highest concentration of terpenoids among the tested residues, and therefore, this substrate is the most representative for confirming that the precision of the LPG method for the extraction of terpenes achieved high purity and high concentrations. The LPG method displays high affinity for terpenoids, resulting in clean extractions without unwanted compounds that may exhibit waxy characteristics. This method resulted in little decomposition of the compounds compared to the methods that employ heating. Therefore, this method should be further investigated for industrial uses with terpenes as well as other compounds with similar characteristics, such as aroma compounds and essential oil constituents. This method could be applied as an intermediate step in processes targeted at recuperation of terpenes. In addition, this approach can be easily applied to laboratory extractions.

Acknowledgements

Authors wish to thank the Fundação Araucária (Conv. 490/2010, Proj. 15637), for providing financial support to carry out this work.

References

[1] L.Wang, C. L.Weller, Recent advances in extraction of nutraceuticals from plants, Trends in Food Science & Technology 17 (2006) 300-312.

[2] Y. Yang, B. Kayan, B. Neval, B. Pate, C. Baker, A. M. Gizir, Terpene degradation and extraction from basil and oregano leaves using subcritical water, J. Cromatography A 1152 (2007) 262-267.

[3] G. W. Mcgraw, R. W. Hemingway, L. L. Ingram Jr., C. S. Canady, W. B. Macgraw, Thermal Degradation of Terpenes: Camphene, Δ^3-Carene, Limonene, and α-Terpinene, Environmental Science Technology 33 (1999) 4029-4033.

[4] N. Lebovka, E. Vorobiev, F. Chemat, Enhancing Extraction Processes in the Food Industry, CRC Press, 2011, 570 p.

[5] T. Moslovec, S. Jokić, D. Subarić, K. Aladić, J. Vukoja, N. Prce, Pressing and supercritical CO2 extraction of Camelina sativa oil, Industrial Crops and Products 54 (2014)122-129.

[6] M. M. Cunico, O. G. Miguel, M. D. Miguel, J. L. S. Carvalho, D. P. Montrucchio, J. L. Ferreira. J. S. Oliveira, Extração de esteróides em frutos de Ottonia martiana MIQ., Piperaceae, com Gás Liquefeito, Química Nova. 26 (2003) 803-806.

[7] J. R. F. Silva, K. C. Cantelli, M. B. A. Soares, M. V. Tres, D. Oliveira, M. A. Meireles, J. V. Oliveira, H. Treichel, M. A. Mazutti, Enzymatic hydrolysis of non-treated sugarcane bagasse using pressurized liquefied petroleum gas with and without ultrasound assistance, Renewable Energy 83 (2015) 674-679.

[8] A. Arce, A. Pobudkowskal, O. Rodríguez, O. Soto, Citrus essential oil terpenless by extraction using 1-ethyl-3-methylimidazolium ethylsulfate ionic liquid: Effect of the temperature, Chemical Engineering J. 133 (2007) 213-218.

[9] S. Diaz, S. Espinosa, E. A. Brignole, Citrus peel oil deterpenation with supercritical fluids optimal process and solvent cycle design, J. Supercritical Fluids 35 (2005) 49-61.

[10] A. R. Yadava, A. S. Chauhan, M. N. Rekha, R. S. Ramteke, Flavour quality of dehydrated lime [Citrus aurantifolia (Christm.) Swingle], Food Chemistry 85 (2004) 59-62.

[11] K.Fisher, C. Phillips, Potential antimicrobial uses of essential oils in food: is citrus the answer?, Trends in Food Science & Technology 9 (2008) 156-164.

[12] A. U. Mahmood, J. Greenman, A. H. Scragg, Orange and potato peel extracts: Analysis and use as Bacillus substrates for the production of extracellular enzymes in continuous culture, Enzyme and Microbial Technology 22 (1998)130-137.

[13] E. Kupcinskiene, A. Stikliene, A. Judzentiene, The essential oil qualitative and quantitative composition in the needles of Pinus sylvestris L. growing along industrial transects, Environmental Pollution 155 (2008) 481-491.

[14] G. Semiz, J. Heijari, K. Isiki, J. K. Holopainen, Variation in needle terpenoids among Pinus sylvestris L. (Pinaceae) provenances from Turkey, Biochemical Systematics and Ecology 35 (2007) 652-661.

[15] S.K. Yoo, D. F. Day, Bacterial metabolism of and -pinene and related monoterpenes by Pseudomonas sp. strain PIN, Process Biotechnology 37 (2002) 739-745.

[16] H. Kofujita, K. Ettyu, M. Ota, Characterization of the major components in bark from five Japanese tree species for chemical utilization, Wood Science and Technology 33 (1999) 223-228.

[17] C. S. Ku, M. Sathishkumar, S. P. Mun, Binding affinity of proanthocyanidin from waste Pinus radiate bark onto proline-rich bovine Achilles tendon collagen type I, Chemosphere 67 (2007) 1618-1627.

[18] A. Nogueira, L. D. Santos, C. Paganini, G. Wosiacki, Avaliação da fermentação alcoólica do extrato de bagaço de maçã, Semina: Ciências Agrárias 26 (2005) 187-194.

[19] C.Villatoro, R. Altisent, G. Echeverría, J. Graell, M. L. López, I. Lara, Changes in biosynthesis of aroma volatile compounds during on-tree maturation of 'Pink Lady®' apples, Postharvest Biology and Technology 47 (2008) 286-295.

[20] G. Echeverría, J. Graell, I. Lara, M. L. López, Physicochemical measurements in 'Mondial Gala®' apples stored at different atmospheres: Influence on consumer acceptability, Postharvest Biology and Technology 50 (2008) 135-144.

[21] M. N. Shashirekha,, R. Baskaran, L. Jaganmohan, Influence of processing conditions on flavour compounds of custard apple (Annona squamosa L.), LWT - Food Science and Technology 41 (2008) 236-243.

[22] J. S. Oliveira, Extrator para Operação com Gases Liquefeitos, Brazilian Patent PI 9804199-1, May 23, 2000.

[23] Instituto Adolfo Lutz, Normas Analíticas do Instituto Adolfo Lutz (Vol 1: Métodos Físico-Químicos para Análise de Alimentos), 4th ed., São Paulo, 2008, pp.117.

[24] L. V. Silva, S. M. Constâncio, M. F. Mendes, G. L. V.Coelho, Extração de Óleo Essencial da Pimenta Rosa (Schinus molle) usando Hidrodestilação e Soxhlet, in: Proceedings of VI Congresso Brasileiro de Engenharia Química em Iniciação Científica, Campinas, 2005.

[25] J. L. Costa, A.L L. Silva, M. C. J. Bier, G. E. Brondani, A. L. Gollo, L. A. J. Letti, E. A. L. Erasmo, C. R. Soccol, Callus Growth Kinetics of Physic Nut (Jatropha curcas

L.) and Content of Fatty Acids from Crude Oil Obtained In Vitro, Applied Biochemistry and Biotechnology 176 (2015) 892-902.

[26] J. Demyttenaere, J. Vanorschelde, N. Kimpe, Biotransformation of (R)-(+)- and (S)-(−)-citronellol by Aspergillus sp. and Penicillium sp., and the use of solid-phase microextraction for screening, J Chromatography 27 (2004) 137-146.

[27] NIST 98 Library with Search Software (Version 1.7), August 1999, National Institute of Standards and Technology, Gaithersburg Md, USA.

[28] R. P. Adams, Identification of Essential Oil Components by Gas Chromatography/Mass Spectrometry, Carol Stream, 2007, 803 p.

[29] S. Zhao, D. Zhang, Supercritical CO2 extraction of Eucalyptus leaves oil and comparison with Soxhlet extraction and hydro-distillation methods, Separation and Purification Technology 133 (2014) 443-451.

[30] C. G. Lopresto, F. Petrillo, A. A. Casazza, B. Aliakbarian, P. Perego, V. Calabro, A non-conventional method to extract D-limonene from waste lemon peels and comparison with traditional Soxhlet extraction, Separation and Purification Technology 137 (2014) 13-20.

[31] D. N. Krassimir, F. M. Tremblav, N. J. Fenton, A. Alexandrov, Structure of Pinus nigra Arn. populations in Bulgaria revealed by chloroplast microsatellites and terpenes analysis: Provenance tests, Biochemical Systematics and Ecology 34 (2006) 562-574.

[32] L. F. Barnola, A. Cedeño, M. Hasegawa, Intra-individual variations of volatile terpene contents in Pinus caribaea needles and its possible relationship to Atta laevigata herbivory, Biochemical Systematics and Ecology 25 (1997) 707-716.

[33] L. Danielski, G. Brunner, C. Schwänke, C. Zetzl, H. Hense, J. P. M. Donoso, Deterpenation of mandarin (Citrus reticulata) peel oils by means of countercurrent multistage extraction and adsorption/desorption with supercritical CO2, J. Supercritical Fluids 44 (2008) 215-324.

[34] P. B. Gomes, V. G. Mata, A. E. Rodrigues, Production of rose geranium oil using supercritical fluid extraction, J. Supercritical Fluids 41 (2007) 50-60.

Capítulo 3 - Biotransformation of limonene by an endophytic fungus using synthetic and orange residue-based media

Artigo publicado no periodico Fungal Biology 121, 2(2017) 137-144

ABSTRACT

Aroma and fragrances have high commercial value for use in food, cosmetics and perfumes. The biotransformation of terpenes by microorganisms represents an attractive alternative method for production of flavorings. Endophytic fungi offer a great potential for the production of several groups of compounds; however, few studies have evaluated the biotransformation of limonene. Following preliminary studies on the biotransformation of limonene, submerged fermentation was carried out using an endophytic fungus isolated from *Pinus taeda* and identified as *Phomopsis sp*. The presence of several biotransformation products was detected and identified by mass spectrometry (GC-MS). The studied strain showed a divergent metabolic behavior, as compounds of interest such as α-terpineol, carvone, and limoneno-1,2-diol were produced under different conditions. In addition to the minor metabolites terpinen-4-ol, menthol and carveol, this strain also produced major metabolites, including 0.536 g/L carvone and 2.08 g/L limonene-1,2-diol in synthetic medium and 2.10 g/L limonene-1,2-diol in a natural orange extract medium with single fed-batch, while the cyclic fed-batch resulted in concentrations less than 1 g/L. Therefore, our study produced a wide variety of limonene derivatives at a high concentration using a natural medium and a newly isolated endophytic fungal strain.

Keywords: Limonene, Biotransformation, Aroma, Endophytic fungus, Limonene-1,2-diol.

1. Introduction

A recent report by Allied Market Research (Ashish, 2014) forecasts that the global flavors market will reach $15.2 billion by 2020 and is expected to grow at a compounded annual growth rate of 4.3% from 2015 to 2020. The growth outlook for natural flavors remains strong, while synthetic flavors may witness negative growth.

Limonene is a naturally occurring cyclic monoterpene that is commonly found in the rinds of citrus fruits such as grapefruit, lemons, limes, and particularly, oranges (Pérez-Mosqueda et al., 2015). Some notable derivatives of limonene are perillyl alcohol, carveol, carvone, α-terpineol and menthol. Carvone- and menthol-flavored compounds are used extensively in the food industry (Maróstica Junior and Pastore, 2007). The

biotransformation of limonene using natural media has been reported in a few cases. Some authors have achieved the biotransformation of limonene by extraction of the orange essential oil, resulting in the production of α-terpineol (Badee et al., 2011) and α-terpineol and perillyl alcohol (Maróstica Jr and Pastore, 2007).

Endophytes are microorganisms that reside asymptomatically in the tissues of higher plants and are a promising source of novel organic natural metabolites, such as enzymes and secondary metabolites that exhibit a variety of biological activities (Pimentel et al., 2011; Corrêa et al., 2014). Molina et al. (2012) suggested that the endophytic microorganisms represent a potential source of natural products for medicinal, agricultural and industrial uses, such as antibiotics, anticancer agents, biological control agents, and other bioactive compounds.

The endophytic fungi present a great potential for the production of several groups of compounds with different applications such as menthol, phenylethyl alcohol and 3-hydroxypropionic acid (Qadri et al., 2015); mammalian metabolites of a natural iridoid entiopicroside (Zeng et al., 2014); pinoresinol diglucoside, pinoresinol monoglucoside, and pinoresinol (Zhang et al., 2015); melanin pigment (Suryanarayanan et al., 2004) and products from the degradation of sinapic acid (Xie et al., 2016). Despite this potential of the endophytes, their use has not yet been assessed.

This study investigated the production of bioaromas by the endophytic fungus *Phomopsis* sp. through the biotransformation of limonene and fermentation using a natural medium instead of a synthetic one.

2. Material and Methods

2.1 Microorganisms and identification

A strain of fungus isolated from the bark of *Pinus taeda* and previously shown to be resistant to limonene and to possess the ability to metabolize limonene was used (Bier et al., 2011). The isolated endophyte strain was identified based in the sequences obtained for the ribosomal DNA Internal Transcribed Spacer (ITS) region. BLAST searches were carried out in the NCBI database (GenBank, NCBI, USA). The sequence data were collected using the software *Data Collection* (v. 1.0.1 Applied Biosystems).

2.2 Inoculum type

Three different inocula were prepared using potato dextrose agar (PDA), orange extract medium or sporulation medium for *Phomopsis* sp., as previously described by Nithya and Muthumary (2010).

2.2.1 Mycelial suspension from PDA

The fungus was cultivated in an Erlenmeyer flask containing 50 mL of potato dextrose agar (PDA) incubated at 30° C for 168 h. The mycelia suspension was prepared by adding 25 mL of sterile distilled water and Tween 80, followed by magnetic stirring. The inoculum volume was maintained at 3 mL for each 40 mL of synthetic medium (table 1) or orange extract medium.

Table 1: Composition of the mineral medium

Components*	**Concentration (g/L)**
$(NH_4)_2SO_4$	5.0
$(NH_4)_2HPO_4$	1.42
NaCl	0.50
$MgSO_4.7H_2O$	0.40
$CaCl_2$	0.60
KCl	2.15
$FeSO_4.7H_2O$	0.01
$ZnSO_4$	0.01
$CuSO_4$	0.01
Glucose	10

*Adapted from Bicas and Pastore (2007)

2.2.2 Orange extract medium

The orange extract medium was prepared by the addition of 10 mL of water/g of dry orange waste, and the mixture was placed in boiling water for 20 min. The resulting extract was filtered by simple filtration through nonwoven fabric. The filtrate (40 mL) was transferred to 125-mL Erlenmeyer flasks. After the addition of ammonium sulfate (5 g/L), the medium was autoclaved for 15 minutes at 121 °C. The medium should contain 3.1% ± 0.24 of reducing sugar according to the Somogyi-Nelson method (Somogyi, 1952) and 2.52 g/L limonene, which was verified by gas chromatography (GC). Table 2 shows the percent composition of the orange waste used in the medium.

Table 2: Physicochemical composition of the Orange waste performed according to the analytical standards of Instituto Adolfo Lutz (2008)

Constitution (%)	Orange waste
Moisture	14.2 ± 0.14
Protein	5.69 ± 0.20
Ashes	3.20 ± 0.12
Lipids	2.89 ± 0.15
Carbon	42.25
Nitrogen	1.33
Hydrogen	6.16
Sulfur	0.00
(R)-(+)-Limonene	5,36 ± 0,46

2.2.3 Sporulation medium

To test its teleomorph form (*Diaporthe)*, the fungus was grown in Erlenmeyer flasks containing 250 mL of medium consisting of 1% glucose, 0.5% peptone, 0.2% NaCl, and 3% glycerol (Matsumae, 1963; Omura, 1976) and incubated for 20 days at 25°C. The mycelium suspension was used as pre-inoculum for this medium.

2.3 Biomass Determination

The biomass of the fungus in submerged fermentation was calculated from the dry weight after vacuum filtration with a 0.22-µm membrane filter.

2.4 Limonene Biotransformation

Experiments were performed with different inoculum types in 125-mL Erlenmeyer flasks containing 40 mL of mineral medium (Table 1) supplemented with 1% (v/v) (R)-+-limonene (Sigma 97%) and 10 g/L glucose. The fermentation was allowed to continue for ten days under continuous agitation at 120 rpm at 30° C. Two negative controls were incubated in parallel under the same conditions. In the first negative control, the growth medium consisted of a mineral medium without the addition of limonene. The second control had fermentation medium but without inoculation.

After preliminary experiments, more biotransformation experiments (7 days at 30ºC, 120 rpm) were performed with two different media (orange residue extract and mineral medium) under two different operational conditions (single fed-batch and cyclic fed-batch). The process was carried out in Erlenmeyer flasks with screw caps (125 mL). The

limonene was added to the medium after the mycelial growth (96 h for orange residue extract and 144 h for the mineral medium), and then the flasks were tightly closed to prevent the loss of volatile compounds. To test the effect of limonene, 1% (v/v) limonene was added in mineral media and 0.5% in the orange medium in the single fed-batch. In the cyclic fed-batch, it was added to a concentration of 0.25% (v/v) in the mineral medium and 0.125% in the orange medium over four days. Fermentation was allowed to proceed for three more days after the addition of limonene in the cyclic fed-batch. All experiments were performed in duplicate.

2.5 Extraction from the fermented media

The extraction methodology used was adapted from Shashirekha et al. (2008). Briefly the fermented media were extracted with 80 mL of dichloromethane/n-pentane (1:1). The extraction procedure started with 30 min of contact with the solvent in the Erlenmeyer flasks containing the fermented media. Afterwards, the solvent was separated from the aqueous phase using a separatory funnel. The solvent phase was passed through a column containing anhydrous sodium carbonate and then concentrated to 3 mL in a flat-bottomed flask coupled to a Vigreux column. The concentration occurred under vacuum at 35°C using a warming plate set at 55°C.

2.6 Analysis of Volatiles

The analysis method was as described by Bier et al. (2016). A gas chromatograph GC-17A (Shimadzu) equipped with a flame ionization detector, HP-5 column (30 m x 0.32 mm) and nitrogen as the carrier gas was used. The injector temperature was 250 °C, the detector was set at 280 °C, and the oven temperature was initially set at 40 °C for 2 min, increased by 5 °C/ min to 150 °C, increased by 10 °C/ min to 170 °C, and then further increased by 30 °C/ min to a final temperature of 250 °C, which was held for 2 minutes.

The split ratio was 1:40. The concentration of limonene-1,2-diol was calculated using (1S-2S-4R)-+-limonene-1,2-diol (purity ≥ 97% Aldrich) as an external standard. The other compounds were calculated as limonene equivalents ((R)-(+)-limonene - 97% Sigma).

2.6.1 Determination of the compounds by GC-MS

The analysis of volatile compounds in the extract was performed using a Varian GC-MS 3800/Saturn CP 2000 equipped with a CP-Sil 8 CB column (30 m x 0.25 mm). The initial temperature was 60 °C which was increased by 3 °C/min to 250° C. The split was 1/200. The scan range was 30-500 m/z. The identification of peaks was achieved by comparison of the arithmetic index (AI) of the compounds to the specific literature (Adams, 2010) in addition comparison to the MS standards of the National Institute of Standard and Technology (NIST 98, 1999). The identity of the compounds was confirmed by comparing the retention times with retention times of the of the following standards: (-)-terpinen-4-ol (Aldrich, ≥ 95%), D-carvone (Sigma-Aldrich, ≥ 96%), (1S-2S-4R)-+-limoneno-1,2-diol (Aldrich, ≥ 97%), terpineol (Aldrich, 99.5%), (-)-carveol (Sigma-Aldrich, 97%) and 1R-2S-5R-(-)-menthol (Sigma-Aldrich, 99%) in gas chromatography.

3 Results

3.1 Identification

The strain used in the experiments is available from and currently maintained by the Culture Collection of the Laboratory of Biotechnological Processes (LPBI) at the Federal University of Paraná (UFPR). The obtained rDNA ITS sequence was deposited in GenBank under the accession number KY113119.

BLAST searches showed 100% similarity to sequences obtained for species of *Phomopsis* (teleomorph: *Diaporthe sp*.). The anamorph was used in the biotransformation experiments; hence, the fungus will be treated as a *Phomopsis sp*. Species identification was not carried out.

3.2 Inoculum tests

Three types of fungal inocula were screened during the 240 h of cultivation in the mineral medium containing 1% (400 μL) (R)-(+)-limonene. The sporulation medium inoculum was tested in order to analyze the bioconversion capacity of spores produced by *Diaporthe* sp. (teleomorph form of *Phomopsis*). The orange extract medium was tested in order to acclimatize the fungus for the fermentation process. The results obtained showed that the fungus strains exhibited different metabolic profiles that was

different for each of the inocula tested (Table 3). The fungus produced by the inoculum grown in the sporulation medium produced (-)-carvone as its major product, while the mycelial suspension cultured in PDA produced α-terpineol as the major product. Fungus grown in the orange extract produced high concentrations of limonene-1,2-diol.

Table 3: Maximum concentration and time of highest production of the most significant metabolites observed in mineral medium with each inoculum in mg/L.

Compound/Inoculum	Sporulation medium	Mycelial suspension	Orange extract
(R)-(−)-Carvone	208.50 - 144 h	127.71 – 144h	39.53 – 48h
Terpinen-4-ol	29.45 – 96h	30.14 – 144 h	28.30 – 96h
α-terpineol	26.53 – 96h	587.60 – 48h	44.90 – 96h
(1S,2S,4R)-(+)-Limonene-1,2-diol	173.04– 144h	107.55 – 144h	962.3– 144 h
Cis-Carveol	146.98 – 96h	133.14 – 144h	43.18 – 48h
Trans-Carveol	107.05 – 96h	102.38 – 144h	47.94 – 48h

3.3 Limonene Biotransformation

The fungal strain *Phomopsis* sp. presented some compounds of interest such as carvone and carveol, which were detected by headspace analysis. After 7 days of culture, extracts of the resulting mycelia contained several compounds resulting from the biotransformation of limonene. The transformed compounds were identified by mass spectrometry and quantified by GC analysis (Table 4) and included trans-carveol, cis-carveol, (-)-carvone and limonene-1,2-diol. Other compounds resulting from the fermentation process were also detected by mass spectrometry.

Table 4: GC-MS of volatile compounds produced by *Phomopsis sp* in mineral medium containing 1% of limonene

Compound	CAS registry number	Experimental AI	Literature AI	Concentration µL / L
Trans-p-mentha-2,8 dien-1ol	7212-40-0	1124	1122	90.70
Cis-p-mentha-2,8 dien-1ol	22771-44-4	1139	1137	37.40
Menthol	89-78-1	1171*	1171	45.33
Terpinen-4-ol	562-74-3	1177*	1176	24.59
α-terpineol	98-55-5	1188*	1190	33.34
1,6-Dihydrocarveol	619-01-2	1197	1193	**
Trans-Carveol	1197-07-5	1217*	1216	132.10
Cis-Carveol	1197-06-4	1230*	1229	50.09

(R)-(−)-Carvone	6485-40-1	1243*	1243	245.10
(1S,2S,4R)-(+)-Limonene-1,2-diol	38630-75-0	-*	-	73.80

*= Confirmed by the standards **= only trace amounts were found.

(R)-(+)-Limonene consumption and limonene-1,2-diol production were monitored during the culture of *Phomopsis* sp. Figure 1 shows the initial concentration of limonene and limonene-1,2-diol at the point of inoculation in the mineral medium.

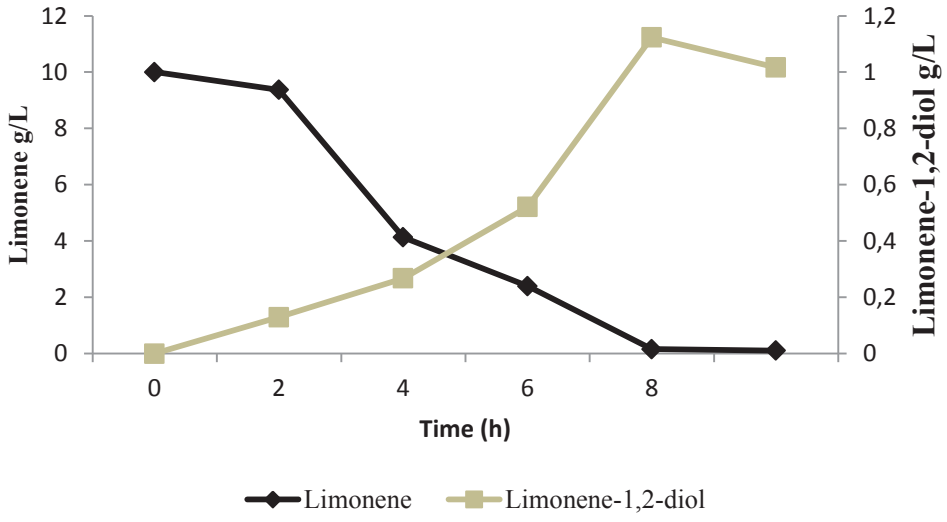


Figure 1: Concentration of limonene and limonene-1,2-diol in mineral media fermentation with 1% glucose.

In this study, one of the most important compounds detected was limonene-1,2-diol, which reached a maximum concentration of 1.12 g/L after 8 days of fermentation. The overall production yield was calculated to be 10.85%.

3.4 Phomopsis sp. Growth

New assays of the biotransformation of limonene were performed by adding limonene in the middle of the log phase of fungal growth. The growth kinetics were studied for *Phomopsis* sp. grown in the orange extract medium and the mineral medium. *Phomopsis* sp. reached the middle of the log phase in the mineral medium and in the orange extract medium after 144 h of growth and after 96 h of growth, respectively (figures 2 and 3).

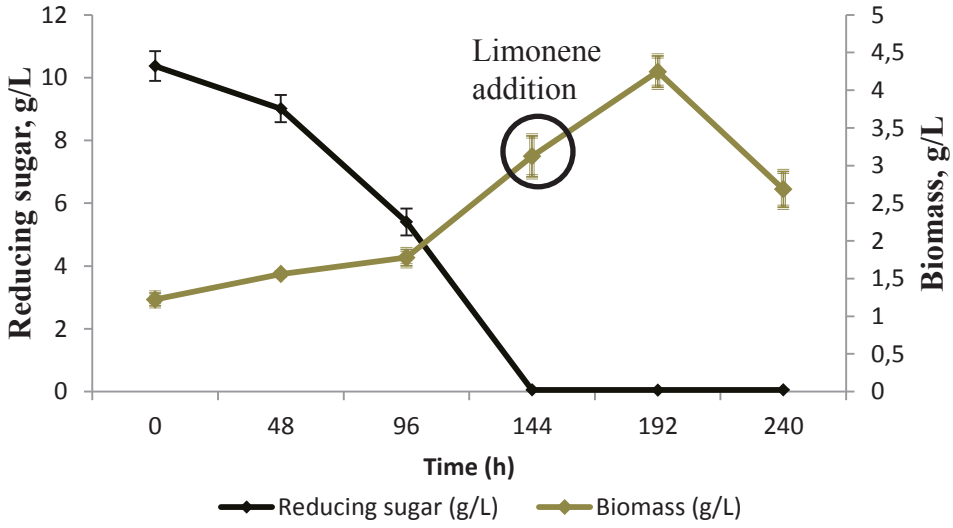


Figure 2: Reducing sugar consumption during the growth of *Phomosis sp*. on mineral medium

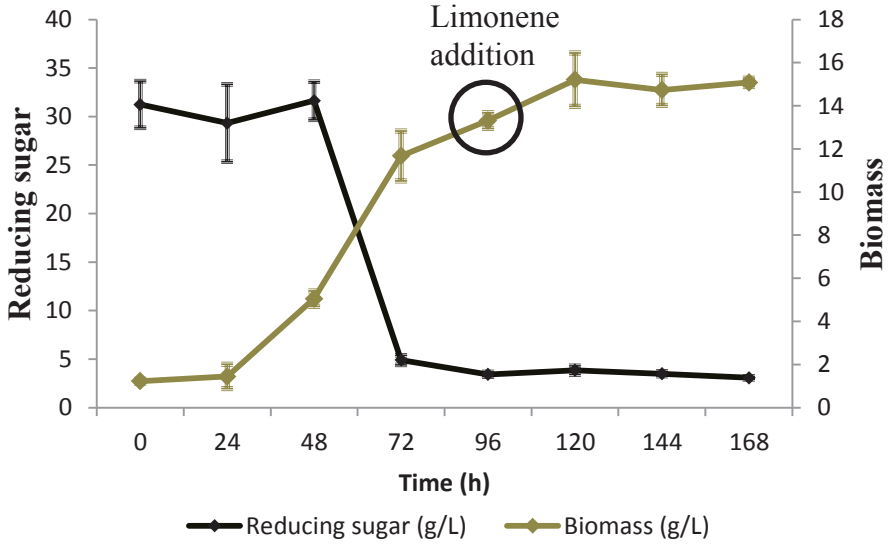


Figure 3: Reducing sugar consumption during the growth of *Phomosis* sp. on orange extract

3.5 Limonene biotransformation in mineral medium

In these experiments, limonene-1,2-diol was the major compound produced during the biotransformation of limonene. Other compounds such as α-terpineol and terpinen-4-ol were present only as minor products and were present at a maximum concentration of approximate 23.64 mg/L and 89.74 mg/L, respectively. The maximum carvone yield was 536 mg/L.

As shown in figure 4, in the single fed-batch fermentation, the concentration of limonene 1,2-diol was approximately 2.08 g/L after 5 days of fermentation in the biotransformation experiments. Within the same duration, the cyclic fed-batch yielded only 0.54 g/L of limonene 1,2-diol.

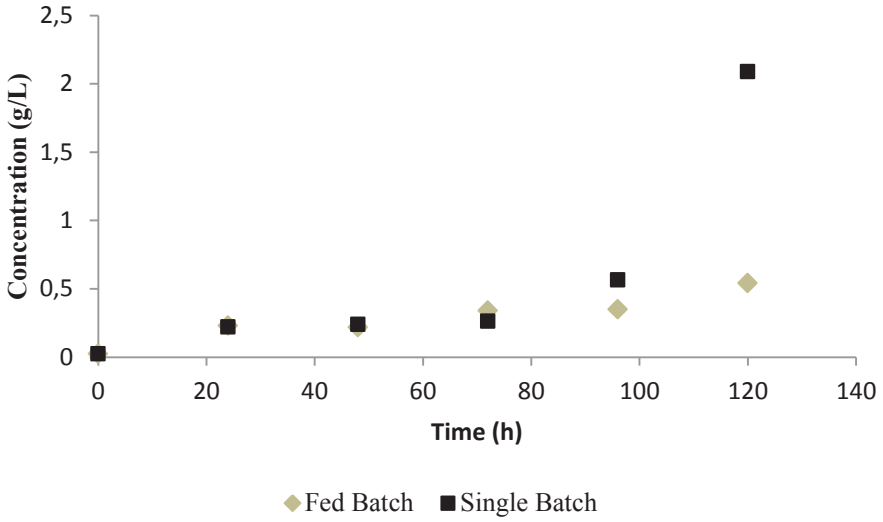


Figure 4: Concentration of limonene-1,2-diol in mineral media in fed and single batch

3.6 Limonene biotransformation in orange extract medium

In Figure 5 the results show the yield of limonene-1,2-diol in orange extract media. The limonene-1,2-diol yield was 2.10 g/L after 6 days of fermentation. The maximum concentration of terpinen-4-ol was 17.01 mg/L, that of α-terpineol was 34.22 mg/L, and that of carvone reached 12.56 mg/L. The cyclic fed-batch yielded 0.49 g/L of limonene-1,2-diol 6 days after the addition of limonene. The concentration of limonene-1,2-diol decreased slowly after the maximum production time, with the same behavior shown in Figure 1.

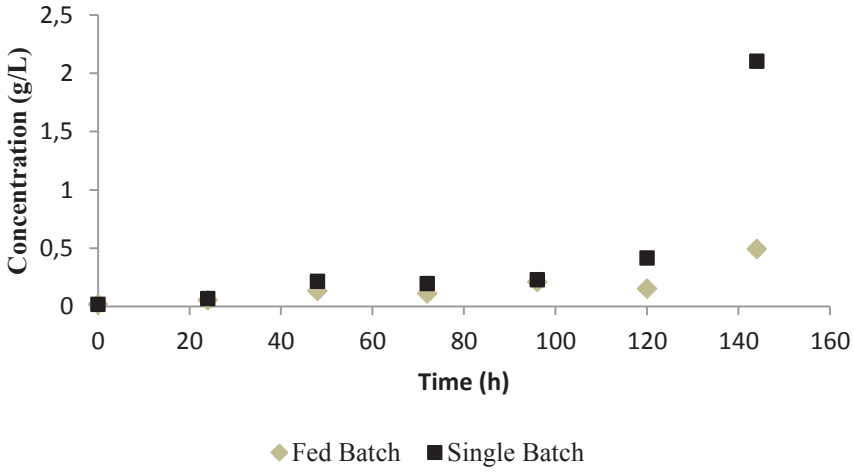


Figure 5: Concentration of limonene-1,2-diol in orange extraction fermentation in fed and single batch

4. Discussion

Phomopsis / *Diaporthe* are known as important endophytic fungi or as plant pathogens; Members of this genus produce a diverse array of secondary metabolites (Abreu et al., 2012; Ferreira et al., 2015).

The inoculum tests of the studied fungi showed changes in the metabolic profiles for each of the inoculum types with regard to the major products obtained. The metabolic changes impacted the production of compounds such as carveol and α-terpineol in terms of the rate and maximum concentration achieved. However, all the compounds were present in each sample as products with lower concentrations. These results are somewhat controversial, given that different metabolic profiles of anamorph and teleomorph of *Phomopsis* sp. are usually not expected, as reported in a comparative study by Isaka et al. (2007) and stated by Samson and Pitt (1990). It is possible that some secondary metabolites are morph related, as stated by Wicklow and Shotwell (1983). Additionally, there are reports in the literature that have associated the production of certain metabolites with sporulation (Calvo et al., 2002; Sorenson et al., 1987). Živković et al. (2012) demonstrated that each isolate of *Diaporthe* nitschke, and their anamorph *Phomopsis* had characteristically distinctive patterns of protein bands.

During limonene biotransformation, it is possible that limonene is metabolized by the fungus strain to produce biomass or for other purposes. Essential oils and terpenoids are

secondary metabolites derived biosynthetically from primary metabolites. Assuming that biotransformation is a process for the attainment of secondary metabolites from cell cultures (Dave et al., 2014), no bioaromas would be produced from this pathway if limonene is incorporated by the microorganism during the primary metabolism. An alternative to prevent the early use of limonene would be to add it into the fermentation medium after the microbial growth is executed, as described by Maróstica Júnior and Pastore (2007) and Demyttenaere et al. (2001). Carvone and α-terpineol are compounds with high value that can be derived from limonene. Carveol and its isomers are in the metabolic pathway of carvone, as shown by Marostica Júnior and Pastore (2007). The production of carvone using limonene as a precursor has been reported in some studies (Carvalho and Fonseca, 2003; Vanek et al., 1999), albeit at low concentrations, and very few such reports were found in the literature.

Despite the significant production of limonene-1,2-diol (1.12 g/L) in the biotransformation experiments, the yield was still low (10.85%). Nonetheless, in spite of the low production yield, the concentration obtained was higher than most reported in the literature (cf. Vanek et al., 1999; Maróstica Jr and Pastore, 2007; Houjin et al., 2006; Adams et al., 2003). The high diversity of metabolic products and the high concentration of limonene added to the medium may explain the production yield obtained.

Most of the literature on the biotransformation of limonene report limonene-1,2-diol as a minor metabolite (Demyttenaere et al., 2001; Carvalho and Fonseca, 2003). Some authors, however, have reported it as the major product in their fermentation. Mukherjee et al. (1973) obtained 1.5 g/L of limonene-1,2-diol using *Cladosporium* sp. Recently, Molina et al. (2015) obtained 3.7 g/L of limonene-1,2-diol using S(-) limonene as a substrate and a strain of *Fusarium oxysporum*. Both studies were performed with filamentous fungi using synthetic medium.

The assays performed after the addition of limonene at the midpoint of the log phase of the fungus growth showed limonene-1,2-diol as the major compound produced by the biotransformation process. Despite the production of minor compounds already

described in the previous steps such as α-terpineol and carvone (Table 3), their concentrations did not increase significantly during the experiments.

The results of the biotransformation in mineral medium are consistent with what was reported by Tai et al. (2016), who determined the best growth phase at which limonene should be added to achieve the best biotransformation. Tai et al. found that the best yield of α-terpineol was obtained when limonene was added during the log phase or at the end of log phase, although addition at any phase yielded some products. Compared to the previous results, it is evident that the fermentation process took less time in these essays. The addition of limonene (Fig. 2) during the log phase of the fungus growth demonstrated good results, especially when compared with the previous results of limonene-1,2-diol production. While the highest concentration (1.12 g/L) was achieved after 7 and 8 days of fermentation (Fig. 1), in this step, it reached 2.08 g/L (single fed-batch in mineral medium). The addition of limonene after the growth of *Phomopsis sp*. Also shortened the biotransformation time.

The results of the biotransformation in orange residue medium are surprisingly quite similar to those of the biotransformation in mineral media in terms of the limonene-1,2-diol yield, reaching 2.10 g/L after 6 days of fermentation. Most of the yields were below that obtained with mineral medium. (-)-Carvone, specifically, reached a concentration of 536 mg/L in the mineral medium, and α-terpineol was an exception, increasing only slightly.

In these experiments, the cyclic fed-batch did not show better results, which was unexpected since some previous studies have achieved good results by adding low concentrations of limonene (Maróstica Jr and Pastore, 2007; Adams et al., 2003). In the literature, most of the limonene biotransformations have been performed using fast-growing bacteria, and fungi have been used less often. No biotransformation of limonene using endophytic fungi has been reported previously. This may explain the low productivity of the cyclic fed-batch. Contrasting this with the mineral medium, the fungus took one more day in the orange medium to reach its maximum production.

Our results not only provide compelling evidence for the potential use of endophytic fungi in the biotransformation of limonene but also make an important contribution to

the field of natural products. Moreover, this biotransformation could be replicated in natural media for the production of limonene-1,2-diol and for other compounds that occur in minor concentrations. The orange extract medium is much more complex in terms of constituents and has approximately 32 g/L of reducing sugar when compared to mineral medium which requires only the 10 g/L. Despite the high carbon/nitrogen ratio in the orange extract medium, all the constituents necessary to the biotransformation process are naturally derived. These results set precedence for the use of natural media to substitute synthetic which not only is expensive but is also a pollutant.

5. Conclusion

A variety of compounds resulted from the biotransformation of limonene suggesting that this strain of *Phomopsis* has the potential to biotransform limonene. This is the first time this process has been described to occur in endophytic fungi. When cultured in a medium containing glucose with the addition of limonene the process yielded volatiles derived from the metabolism of limonene. Compounds such as α-terpineol, carvone and limonene-1,2-diol were particularly prominent as products in the biotransformation. In the mineral medium, the carvone yield was 536 mg/L after 5 days of biotransformation, while α-terpineol attained a maximum yield of 208.5 mg/L in the sporulation inoculum. The maximum yield of limonene-1,2-diol obtained was 2.08 g/L. In the orange residue extract medium, a high concentration of limonene-1,2-diol was successfully obtained, reaching 2.10 g/L after 6 days of biotransformation. Most of the other compounds were, however, produced only in minor quantities, such as carvone (12.56 mg/L) and terpinen-4-ol (17.01 mg/L); an exception was α-terpineol (34.22 mg/L), which appeared at a lower concentration in the mineral medium under the same conditions (23.64 mg/L). Importantly, all these minor compounds were produced under all the fermentation conditions. Variations in the levels of the major fermentation products were observed when the fungus was subjected to different growth conditions such as the sporulation medium, mycelia in suspension, and orange extract inoculum. Furthermore, the point at which the limonene was added impacted the metabolic profile of *Phomopsis* sp.

The best results of biotransformation using *Phomopsis* sp. were obtained in the single fed-batch operation. The cyclic fed-batch fermentations yielded much lower concentrations of the products.

In conclusion, many derivatives of limonene such as carvone, carveol, α-terpineol, terpinen-4-ol and limonene-1,2-diol were successfully obtained through biotransformation. However, the highlight of this study was the verification of the divergent behaviors of *Phomopsis* sp., showing wide metabolic variations between different forms of the fungus; the achievement of significant amounts of carvone using the synthetic medium and; the high yield of limonene-1,2-diol in a natural medium using an isolated endophytic strain.

Acknowledgments

Authors wish to thank the Fundação Araucária (Conv. 490/2010, Proj. 15637) for providing financial support, $LACAUT_{ets}$, Prof. Beatriz Noronha and her group for the analysis support. M.C.J.B. acknowledges the CAPES scholarship.

References

Abreu, LM, Costa, SS, Pfenning, L, Takahashi, JA, Larsen, TO, Andersen, B, 2012. Chemical and molecular characterization of *Phomopsis* and *Cytospora*-like endophytes from different host plants in Brazil. Fungal Biology 116: 249-260.

Adams, A, Demyttenaere JCR, De Kimpe N, 2003. Biotransformation of (R)-(+)- and (S)-(-)-limonene to α-terpineol by *Penicillium digitatum* — investigation of the culture conditions. Food Chemistry 80: 525-534

Adams, RP, 2010. Identification of essential oil components by gas chromatography/mass Spectrometry, 4th edn. Allured Publishing Corporation, Carol Stream.

Ashish, S, 2015. Global flavors (food and beverages) market - size, share, trends, analysis, opportunities, growth, and forecast, 2014 – 2020. Allied Market Research, Portland.

Badee, AZM, Helmy, SA Morsy, NFS, 2011. Utilisation of orange peel in the production of α-terpineol by *Penicillium digitatum* (NRRL 1202). Food Chemistry 126: 849–854

Bicas, JL, Pastore, GM, 2007. Isolation and screening of D-limonene-resistant microorganisms, Brazilian Journal of Microbiology 38: 563-567

Bier, MCJ, Poletto, S, Soccol, VT, Soccol, CR, Medeiros, ABP, 2011. Isolation and screening of microorganisms with potential for biotransformation of terpenic substrates, Brazilian Archives of Biology and Technology 54: 1019-1026

Bier, MCJ, Medeiros, ABP, De Oliveira, JS, Côcco, LC, Costa, JL, Carvalho, JC, Soccol, CR, 2016. Liquefied gas extraction: A new method for the recovery of terpenoids from agroindustrial and forest wastes. The Journal of Supercritical Fluids 110: 97-102.

Calvo, AM, Wilson, RA, Bok, JW, Keller, NP, 2002. Relationship between secondary metabolism and fungal development. Microbiology and Molecular Biology Reviews 66: 447-459

Carvalho, CCR, Fonseca, MMR, 2003. Towards the bio-production of trans-carveol and carvone form limonene: induction after cell growth on limonene and toluene. Tetrahedron: Asymmetry 14: 3925-3931

Corrêa, RCG, Rhoden, AS, Mota, TR, Azevedo, JL, Pamphile, JA, Souza, CGM, Polzeli, MLTM, Bracht, A, Peralta, RM, 2014. Endophytic fungi: expanding the arsenal of industrial enzyme producers. Journal of Industrial Microbiology & Biotechnology 41: 1467-1478 (2014).

Dave, V, Khirwadkar, P, Dashora, K, 2014. A review on biotransformation. Indian Journal of Research in Pharmacy and Biotechnology 2: 1136-1140

Demyttenaere, J, Belleghem, KV, Kimpe, N, 2001. Biotransformation of terpenes by fungi Study of the pathways involved. Journal of Molecular Catalysis B: Enzymatic 11: 265–270.

Ferreira, MC, Vieira, MLA, Zani, CL, Alves, TMA, Junior, PAS, Murta, SMF, Romanha, AJ, Gil, LHVG, Carvalho, AGO, Zilli, JE, Vital, MJS, Rosa, CA, Rosa, LH, 2015. Molecular phylogeny, diversity, symbiosis and discover of bioactive compounds of endophytic fungi associated with the medicinal Amazonian plant *Carapa guianensis* Aublet (Meliaceae). Biochemical Systematics and Ecology 59: 36-44

Houjin, L, Wenjian, L, Chuanghua, C, Yipin, Z, Yongcheng, L, 2006. Biotransformation of limonene by marine bacteria, Chinese Journal of Analytical Chemistry 34: 946-950

Instituto Adolfo Lutz, 2008. Métodos físico-químicos para análise de alimentos, 4th edn. Instituto Adolfo Lutz, São Paulo.

Isaka, M, Palasarn, S, Kocharin, K, Hywel-Jones, NL, 2007. Comparison of the bioactive secondary metabolites from the scale insect pathogens, anamorph *Paecilomyces* cinnamomeus, and teleomorph *Torrubiella luteorostrata*. Journal of Antibiotics 60: 577–581

Maróstica Jr, MR, Pastore, GM, 2007. Production of R-(+)-α-terpineol by the biotransformation of limonene from orange essential oil, using cassava waste water as médium. Food Chemistry 101: 345-350.

Maróstica Júnior, MR, Pastore, GM, 2007. Biotransformation of limonene: a review of the main metabolic pathways. Química Nova 30: 382-387

Matsumae, A, Kamio, Y, Hata, T, 1963. Studies on cerulenin producing strain. Journal of Antibiotics 16: 239-243

Molina, G, Bution, ML, Bicas, JL, Dolder, MAH, Pastore, GM, 2015. Comparative study of the bioconversion process using R-(+)- and S-(–)-limonene as substrates for *Fusarium oxysporum* 152B. Food Chemistry 174: 606–613

Molina, G, Pimentel, MR, Bertucci, TCP, Pastore, GM, 2012. Application of fungal endophytes in biotechnological processes. Chemical Engineering Transactions 27:. 289-294

Mukherjee, BB, Kraidman, G, Hill, ID, 1973. Synthesis of glycols by microbial transformation of Some monocyclic terpenes. Applied Microbiology 25 447-453

Nithya, K, Muthumary, J, 2010. Secondary Metabolite from *Phomopsis sp.* isolated from *Plumeria acutifolia* Poiret. Recent Research in Science and Technology 2: 99-103

NIST 98 Library with Search Software (Version 1.7), 1999. National institute of standards and technology, Gaithersburg, MD, USA.

Omura, S, 1976. The antibiotic cerulenin, a novel tool for biochemistry as an inhibitor of fatty acid synthesis. Bacteriological Reviews 40: 681–697

Pérez-Mosqueda, LM, Trujillo-Cayado, LA, Carrillo, F, Ramirez, P, Muñoz, J, 2015. Formulation and optimization by experimental design of eco-friendly emulsions based on D-limonene. Colloids and Surfaces B: Biointerfaces 128: 127-131

Pimentel, MR, Molina, G, Dionísio, AP, Maróstica Junior, MR, Pastore, GM, 2011. The use of endophytes to obtain bioactive compounds and their application in biotransformation process, Biotechnology Research International 2011: 1-11

Qadri, M, Deshidi, R, Shah, BA, Bindu, K, Vishwakarma, RA, Riyaz-Ul-Hassan, S, 2015. An endophyte of *Picrorhiza kurroa* Royle ex. Benth, producing menthol,

phenylethyl alcohol and 3-hydroxypropionic acid, and other volatile organic compounds. World Journal of Microbiology and Biotechnology 31: 1647-1654

Samson, RA, Pitt, JI, 1990. Modern Concepts in *Penicillium* and *Aspergillus* classification, 1st edn. Springer Science Business Media, New York.

Shashirekha, MN, Baskaran, R, Jaganmohan, L, 2008. Influence of processing conditions on flavour compounds of custard apple (*Annona squamosa* L.). LWT - Food Science and Technology 41 236-243.

Somogyi, M, 1952. Estimation of sugars by colorimetric method. Journal of Biological Chemistry 200

Sorenson, WG, Frazer, DG, Jarvis, BB, Simpson, J, Robinson, VA, 1987. Trichothecene mycotoxins in acrolized conidia of *Stachybotrys atra.* Applied and Environmental Microbiology 53: 1370-1375

Suryanarayanan, TS, Ravishankar, JP, Venkatesan, G, Murali, TS, 2004. Characterization of the melanin pigment of a cosmopolitan fungal endophyte. Mycological Research 108: 974-978.

Tai, Y-N, Xu, M, Ren, J-N, Dong, M, Yang, Z-Y, Fan, G, 2015. Optimisation of α-terpineol production by limonene biotransformation using *Penicillium digitatum* DSM 62840. Journal of Science and Food Agriculture 96: 965-961

Vanek, T, Valterová, I, Vanková, R, Vaisar, T, 1999. Biotransformation of (-)-limonene using Solanum aviculare and Dioscorea deltoidea immobilized plant cells. Biotechnology Letters 21: 625-628

Wicklow, DT, Shotwell, OL, 1983. Intrafungal distribution of aflatoxin among conidia and esclerotia of *Aspergillus flavus* and *Aspergillus parasiticus*. Canadian Journal of Microbiology 29: 1-5

Xie, X-G, Huang, C-Y, Fu, W-Q, Dai, C-C, 2016. Potential of endophytic fungus *Phomopsis liquidambari* for transformation and degradation of recalcitrant pollutant sinapic acid. Fungal Biology 120: 402-413

Zeng WL, Li WK, Han H, Tao YY, Yang L, Wang ZT, Chen KX, 2014. Microbial biotransformation of gentiopicroside by the endophytic fungus *Penicillium crustosum* 2T01Y01. Applied and Environmental Microbiology 80: 184-192

Zhang, Y, Shi, J, Gao, Y, Yangwu, R, Jiang, H, Che, J, Liu, Y, 2015. Production of pinoresinol diglucoside, pinoresinol monoglucoside, and pinoresinol by *Phomopsis* sp. XP-8 using mung bean and its major components. Applied Microbiology and Biotechnology 99: 4629-4643

Živković, S, Jošić, D, Popović, T, 2012. Characterizantion of *Diaporthe/Phomopsis* spp. from plum trees by dds-page. In: International symposium on current trends in plant protection, Belgrade, Sérvia.

Capítulo 4 - Biotransformation of R-(+)-limonene in solid state fermentation of orange waste by *Phomopsis* sp. and evaluation of the antioxidant activity of the fermentation products

Mário César Jucoski Bier[1], Adriane Bianchi Pedroni Medeiros[1*], Cristine Rodrigues[1], Norbert De Kimpe[2] and Carlos Ricardo Soccol[1]

[1] Department of Bioprocess Engineering and Biotechnology, Universidade Federal do Paraná, R. Francisco H. dos Santos, CEP 81531-990, Curitiba, PR, Brazil

[2] Department of Sustainable Organic Chemistry and Technology Faculty of Bioscience Engineering, Ghent University, Coupure Links 653, B-9000 Gent, Belgium

* Corresponding Author: Adriane Bianchi Pedroni Medeiros

Department of Bioprocess Engineering and Biotechnology, Universidade Federal do Paraná

81531-970, Curitiba PR, Brazil

E-mail: adrianebpm@ufpr.br

Tel.: +55 41 33613697

Fax: +55 41 33613695

ABSTRACT

The aim of this study was the biotransformation of R-(+)-limonene through solid state fermentation by the endophytic fungus *Phomopsis* sp. on a natural medium composed of orange waste. The antioxidant potential of the fermented orange waste was evaluated. The fermented media was extracted using liquefied petroleum gas and the presence of several biotransformation products was detected and identified by mass spectrometry (GC-MS) and quantified by gas chromatography (GC). The antioxidant activity of the compounds formed was analyzed by the ORAC method, DPPH method and the CUPRAC method. Compounds such as limonene-1,2-diol, α-terpineol, (-)-carvone, α-tocopherol, dihydrocarveol and valencene were obtained through the fermentation process, and most of them, have already been related to antioxidant activity. The highest concentration of limonene-1,2-diol produced was 3.02 g/kg of dry substrate and 0.72 g/kg of α-terpineol. According to the results obtained with DPPH method, the extract of the fermented media presented 20.17% of antioxidant activity in comparison to 12.11% of the orange waste extract in a concentration of 4%, while for the ORAC method the results were 24011.39 μmolTE/g in comparison to 5226.45 μmolTE/g and in the CUPRAC method 538.05 mg TE per g of dry extract in comparison to 168.27 TE per g of dry extract, proving that the fermentation process increased the antioxidant potential of the orange waste extract.

Keywords: Limonene, Endophytic fungus, Antioxidant activity, Solid state fermentation, Biotransformation.

1. Introduction

The global forecast for the orange production in the world was estimated to 49.6 million tons, while the global forecast to orange juice production for 2016/17 was estimated to 2.0 million metric tons (United States Department of Agriculture, 2017). According to Crizel *et al.* (2013), Mahmood *et al.* (1998) and Fiorentin *et al.* (2010), the orange residue in juice processing, composed of seed, flesh and skin, comprises approximately 50 % of the fruit.

Solid-state fermentation (SSF) has emerged as a potential technology for the production of microbial products such as feed, fuel, food, industrial chemicals and pharmaceutical products. Utilization of agro-industrial residues as substrates in SSF processes provides an alternative avenue and value-addition to these otherwise under- or non-utilized residues (Pandey, 2003; Silva *et al*. 2013). Recently, different microorganisms have

been tested using orange oil in submerged fermentation (Badee *et al.* 2011; Maróstica Jr. and Pastore, 2007). However, some authors have followed a different approach in the search for various products using the orange peel and bagasse (Mantzouridou *et al.* 2015; Nicolini *et al.* 1987; Yang *et al.* 2013; Pourbafrani *et al.* 2010; Santi *et al.* 2014).

Yet, there are no records of the production of limonene derivatives on solid state fermentation and the biotransformation of limonene using natural media is also scarce. Due to the high amount of orange waste available and the advantages of the biotechnological and biotransformation process, the utilization of this residue shows great potential. Citrus fruits have high concentrations of limonene (Arce *et al.* 2007, Diaz *et al.* 2005; Yadava *et al.* 2004; Fisher and Phillips, 2008) and therefore, it would be a great source of limonene for biotransformation processes.

Bacteria have been widely used in bioengineering, but endophyte fungi, as a kind of organism, have not been fully investigated (Wang and Dai, 2011). The endophytic fungi represent a great potential for the production of several groups of compounds with different applications (Qadri *et al.* 2015). However, few articles have been published achieving significant concentrations of aroma compounds and none regarding significant concentrations of limonene derivatives via a biotransformation process. The biotransformation of terpenes is of interest because it allows the production of enantiomerically pure flavor compounds and fragrances under mild reaction conditions (Carvalho and Fonseca, 2006).

Besides the sensorial properties, many studies highlight bioactive properties of the essential oil and orange peel as antifungal activity (Velázquez-Nuñez *et al.,* 2013), anti - inflammatory effects (Gosslau *et al.* 2014), antioxidant (Lu *et al.* 2012; Chen *et al.* 2012), antitumoral (Kaur and Kaur 2015) and pesticide activity (El-Akhal *et al.* 2015). Limonene and its derivatives also have many bioactive properties reported such as antioxidant activity and antigenotoxic activies (Bacanli et *al.* 2015), and inhibition of angiogenesis, metastasis and cell death in human colon cancer cells (Murthy et *al.,* 2012). Among these properties, this article highlights an increase of the antioxidant capacity of the fermented product with respect to the orange residue.

This study aimed at the biotransformation of limonene through solid state fermentation by the endophytic fungus *Phomopsis* sp. on a natural medium composed of orange waste. Regarding the antioxidant activity a comparison between the orange extract and the fermented extract is made.

2. Material and methods

2.1 Microorganism

Phomopsis sp. (*Diaporthe*) was previously selected for resistance to limonene and its ability to metabolize it (Bier *et al.* 2011; Bier *et al*. 2017). The strain is maintained in the Culture Collection of the Laboratory of Biotechnological Processes at Federal University of Paraná (LPBI-UFPR), registered on the World Data Centre for Microorganisms (WDCM). The fungus *Phomopsis* sp. was isolated from the bark of *Pinus taeda*. Its rDNA ITS sequence was deposited in GenBank under the accession number KY113119.

2.2 Orange waste

The orange waste used for the fermentation process (peel and bagasse) was obtained from orange juice extraction in a canteen located at the Federal University of Paraná (Curitiba, PR, Brazil). The solid substrate "in natura" was cut into pieces and dried in an oven with air circulation at a temperature of 60 °C in order to prevent degradation during storage, and to facilitate subsequent milling. The dried material was milled in a Wiley mill knife type and classified granulometrically between 0.8 and 3 mm. The essential oil content in the orange residue was previously determined as 5.36% (Bier *et al.* 2016). Its main terpene composition consisted of 95.32 % of R-(+)-limonene, 0.4% of β-pinene and 0.24% of α-pinene. The physicochemical composition (Table 1) of the orange waste (peel and bagasse) was determined according to the analytical standards of Instituto Adolfo Lutz (2008).

Table 1: Physicochemical composition of the Orange waste according to the analytical standards of Instituto Adolfo Lutz (2008)

Constitution	Orange waste
Moisture (%)	14.2 ± 0.14
Reducing sugar (%)	10.13 ± 0.99
Total sugar (%)	21.13 ± 1.57
Protein (%)	5.69 ± 0.21
Ashes (%)	3.20 ± 0.12
Lipid (%)	2.89 ± 0.15

Table 2: Porcentage of limonene, α-pinene and β-pinene in the extract of orange waste (Bier *et al.* 2016)

R-(+)-Limonene (%)	β-pinene (%)	α-pinene (%)
95.32 ± 0.94	0.4 ± 0.04	0.235 ± 0.005

2.3 Inoculum preparation

Phomopsis sp. was cultivated in Erlenmeyer flasks of 250 mL containing 50 mL of potato dextrose agar (PDA) and incubated at 30 °C for 168 h. The mycelial suspension was prepared adding 25 mL of sterile distilled water under magnetic stirring for 10 minutes. *Phomopsis* sp. showed no spores. The inoculum volume of 3 mL for 40 mL of medium was fixed.

The medium was prepared by adding 10 mL of water/g of dry orange waste and placed in a boiling water bath for 20 min. The extract was filtered and separated into Erlenmeyer flasks of 125 mL containing 50 mL in each.

The inoculum was grown during 5 days at 30 ° C under agitation of 120 rpm in a natural orange medium to which ammonium sulfate (5 g/L) was added. The medium was autoclaved for 15 min at 121 °C. Five mL of the fungi suspension was added to the solid state fermentation medium.

2.4 Solid state fermentation

Experiments were carried out according to the process described by Soccol *et al.,* (2014). The orange peel containing residue and orange bagasse was used as a substrate for solid state fermentation due to its high content of limonene (5.08 %). In Erlenmeyer flasks of 250 mL, 20 g of dried orange residue was placed. The water content was

adjusted to 80% moisture. The initial pH of the medium was adjusted to 6.0. The particle size distribution of the medium was a mixture (1:1, w/w) of particles from 0.8 to 2 mm and 2 mm to 3 mm. The culture media was sterilized by autoclaving at 121 ° C for 15 minutes. The fermentation occurred over a period of 7 days at 30 ° C.

2.5 Optimization

Experimental designs were used to study the main factors that influence the biotransformation of limonene by SSF. The experimental designs were developed using the software Statistica® version 7.0. Tests were carried out to study the effects of pH, inoculum ratio and granulometry of the substrate.

An experimental design (2^3) with three factors and three replicates of the central point was applied. Due to its importance reported in the literature, the following independent variables were studied: pH, inoculum ratio and particle size. Humidity was maintained at 80% due to inefficiency of microorganism growth on less than 80% of moisture. The pH levels studied were: 4 (-1), 5 (0) and 6 (+1), a volume inoculum of 3 mL (-1), 5 mL (0) and 7 mL (+ 1) and a lower granulometry from 0.8 to 2 mm (-1), a mixture of 0.8 to 2.0 and 2.0 to 3.0 mm (0) and from 2.0 to 3.0 mm (+1). A second fractional experimental design, with three factors and three levels (3^{3-1}) was implemented with the same variables, using the values of the previous central points as levels (pH: 5; inoculum volume: 5 mL and granulometry: a mixture of 0.8 to 2.0 and 2.0 to 3.0 mm).

2.6 Extraction procedure

The extraction of terpenoids from the orange residue was performed in a portable extractor equipment (Bier *et al.*, 2016). Liquefied petroleum gas (LPG), a mixture comprising of butane and propane, was used as the extractor solvent. The composition of the gas used is propane (25% ± 5%) and isobutane + n-butane (75% ± 5% w/w). The sample amount was 22 g. Each cycle of extraction used 15 g of LPG (Volcano Isqueiros Ltda., SP, Brazil). The exposure time of the sample material was set to 20 min per cycle at 35ºC. All assays were performed in triplicates.

2.7 Fourier Transform Infrared Characterization (FTIR)

The functional groups in the extracts of the samples of orange waste and its respective fermentation extract were determined by medium Infrared Spectroscopy (FTIR) in a VERTEX 70 (Bruker) equipment with DRIFT accessory with 64 scans, 4 cm^{-1} resolution, without the deletion of atmospheric compensation in the region of 4000-400 cm^{-1}. The samples were previously dried in the oven (35ºC, 24h). In the determination, approximately 20 mg of the sample was mixed with 100 mg of spectroscopic KBr, homogenised and placed in the collected spectra accessories. The bands obtained in the infrared spectroscopy were characterized according to Silvestein *et al*. (1991) and Barbosa (2007).

2.8 Volatile compounds analysis

Volatile compounds present in the extracts were analyzed according to the liquefied gas extraction method used by Bier *et al.* (2016). The equipment used was a GC-17A gas chromatograph from Shimadzu with a flame ionization detector, HP-5 column (30m x 0.32 mm) and nitrogen as the carrier gas. The injector temperature was 250°C, the detector temperature was 280°C, and using a temperature program of the oven initially at 40°C for 2 min and increasing 5°C/min to 150°C. From 150ºC to 170º C there was an increase of 10ºC/min and thereafter an increase of 30º C/min until the final temperature of 250°C, maintaining at this temperature for 2 min. A split ratio of 1:40 was used. The extracts were diluted in n-hexane (> 95%). The results were expressed as percentage of peak area of product compared to the peak area of (R)-(+)-limonene (97% Sigma). The analysis of the fermentation extracts of solid state expressed were determined in g/kg substrate based on a standard curve of (1S-2S-4R)-+-Limonene-1,2-diol (≥ 97% Aldrich).

The identification of the volatile compounds was performed in a gas chromatograph coupled with a mass detector (GC-MS) Shimadzu TQ 8040 equipped with a column DB-5 (30 m x 0.32 mm) and triple quadrupole detector. The auto-sampler was an AOC-5000 and the volume injected was 1 μL. The solvent cut was set at 3 min. The scan range was 30 to 400 m/z. The injector and column parameters were the same as used for the gas chromatography analysis. The identification of extract components was

evaluated by comparison with the MS standards of the National Institute of Standard and Technology (NIST14, 2014). All analysis were performed for each of the triplicates. The results were also validated after verification with retention indices in gas chromatography of the standards of D-carvone (Sigma-Aldrich ≥ 96 %), (1S-2S-4R)-+-Limoneno-1,2-diol (Aldrich ≥ 97%), terpineol (Aldrich, 99.5%) and (−)-carveol (Sigma-Aldrich, 97%).

2.9 Determination of the antioxidant activity using the DPPH method

The antioxidant activity of the fermented samples (100 µL), orange waste extract, R-(+)-limonene and limonene-1,2-diol were determined using 1.4 mL of 0.1 mM of DPPH (2,2-diphenyl-1-picrylhydrazyl, Sigma-Aldrich) solution.. This methodology was adapted from Sarrou *et al.* (2013). The DPPH solution was prepared after dilution in 100 ml of methanol and used as the reagent to prepare the standard curves BHA (butylated hydroxyanisole) and ascorbic acid. The IC50 of the extract obtained from the fermented orange residue was determined. The other samples were compared at the concentration of 4%. The analyses were performed in triplicate in a spectrophotometer at 517 nm. A sample containing only methanol was used as the blank.

2.10 Determination of the antioxidant activity using the CUPRAC method

The CUPRAC method (cupric ion reducing antioxidant capacity) was adapted from the test described by Apak *et al.* (2007). The absorbance was measured at 450 nm using the spectrophotometer. The antioxidant potential of R-(+)-limonene, limonene-1,2-diol, orange waste extract and the fermented extracts was measured with Trolox equivalents (TEAC values). The statical significance was verified by the Tukey's method for multiple comparison. Assays that showed statiscal difference from the control samples were labeled as B. If another assay was different from B and the control, it was grouped as C.

2.11 Determination of the antioxidant activity using the ORAC method

The Oxygen Radical Absorbance Capacity Assay (ORAC) was performed according to Zulueta et *al.* (2009). The analysis was performed in a 96 well microplate with the addition of 50 µL of the samples (R-(+)-limonene, limonene-1,2-diol, orange waste extract and fermented extract), 50 µL of fluorescein sodium salt (Sigma) at 78 nM in

phosphate buffer 75 mM, pH 7,0 and 25 µL of AAPH (2,2'-Azobis(2-amidinopropane) dihydrochloride, 97%, Aldrich) – 221 mM in phosphate buffer, daily prepared. The microplater reader used was a TECAN *infinite*® 200MPRO. The sample was exposed to an excitation wavelength of 485 nm and the emission wavelength was 535 nm for 30 minutes (intervals of 60 seconds). The reaction occurred at 37 ºC ± 0.5. The Trolox standard was prepared at concentrations ranging from 3.25 µmol/L to 100 µmol/L. The data were analyzed with Microsoft Excel. The area under the curve (AUC) was calculated as:

$$AUC = 0.5 + f1/f0 + ... fi/f0 + ... + f29/f0 + 0.5(f30/f0)$$

where $f0$ is the initial fluorescence reading at 0 min and fi is the fluorescence reading at time i. The net AUC is obtained by subtraction of the AUC of the blank from that of the sample. The relative Trolox equivalent ORAC value is calculated as:

Relative ORAC value = CTrolox [(AUCsample - AUCblank).k / (AUCTrolox - AUCblank)]

where CTrolox is the concentration of Trolox and k is the sample dilution factor.

2.12 Determination of total phenolics

Total phenolic content was measured using Folin-Ciocalteu (Sigma-Aldrich, 2 mol/L) spectrophotometric method (Singleton and Rossi, 1965; Song *et al.* 2010), using gallic acid (Vetec 98%) for the calibration curve. All tests were performed in triplicate, and the results were presented as gallic acid equivalents (mg/g extract). The sample was diluted to 1:250, and 0.5 mL was reacted with 0.2 mol/L of the Folin-Ciocalteu reagent for 5 minutes. Afterwards, 2 mL of sodium carbonate (7.5% w/v) was added to the reaction mixture. The absorbance readings were performed at 760 nm after incubation at 37 ºC for 60 minutes.

3. Results and discussion

The results obtained by the Infrared Characterization of the orange waste extract and the fermentation extract showed a large variety of bands for both of the samples (figure 1). There is a large band between the 3600-3200 cm^{-1} and another between 2965-2960 of

both extracts. The spectrum obtained has much in similar with the one showed by Reda *et al.* (2005) with seeds of lime, as the bands between 3600-3200 cm^{-1} (hydroxyls of carbonyls), 2965-2864 cm^{-1} (C-H bonds), 1728 cm^{-1} (absorption of carbonyl esters) and 1465 cm^{-1} (C-H bonds), which presence lead to the high concentration of terpenes and some esters in the orange essential oil.

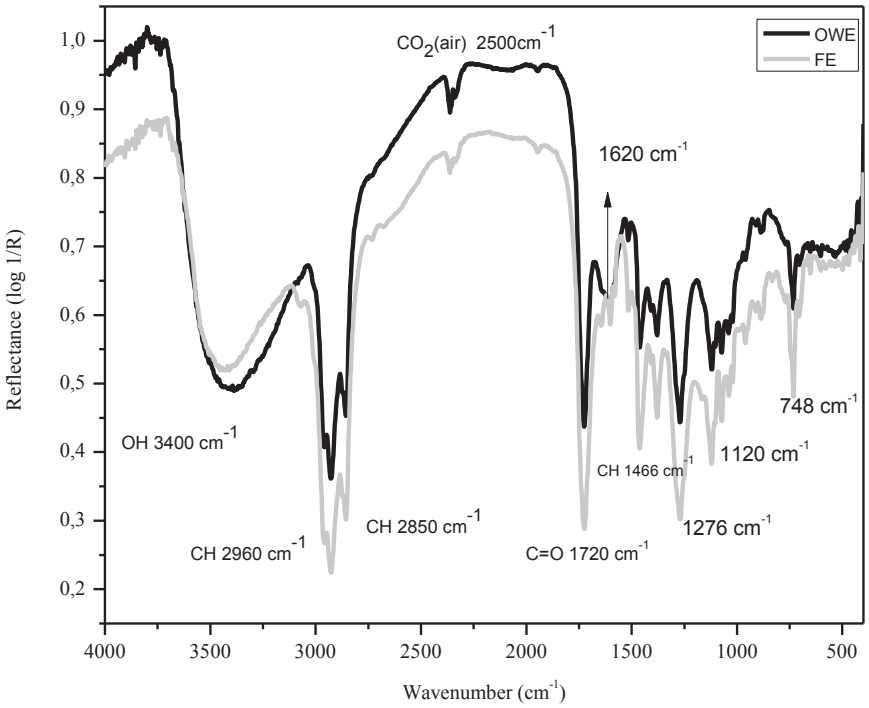


Figure 1: Infrared absorption Spectrum of the orange waste extract (OWE) and fermentation extract (FE).

Many bands can be seen from 1600 to 700, but a few of them are not found in the orange waste extract. While most of them are related to the presence of some functional groups such as acids, esters, alcohols and hydrocarbons, some of the bands absent in the orange waste extract are related to the presence of nitrogen compounds such as amines (1647 cm^{-1}; 700 cm^{-1}) as seen in the table 3. The presence of nitrogen compounds in the fermentation extract may occur due to the fermentation process and can be related to the

production of aromas, as verified by Hue et al. (2014). The presence of amines in fermentations is explained by Smit *et al.* (2012) by the amount of amino acid precursors in the medium and the presence of decarboxylase positive microorganisms.

Table 3: Bands identified in the spectrum of the orange waste extract and fermentation extract.

Bands (cm^{-1})		
OWE	**FE**	**Description**
3400	3425	Stretching vibrations O–H/N-H
2965	2960	Stretching vibrations CH
2930	2930	Asymemtrical stretching vibrations CH_3
2864	2855	Symmetric vibration CH_3 vibrations
1728	1728	Vibration stretching C=O
-	1647	Deformation NH+C=O Stretching vibration (amide)
1600	1600	O-H bending vibration
-	1583	Stretching C=O or NH (amina)
1520	1520	Bending Vibration O-H/N-H
1465	1465	Bending Vibratios C-H
1410	-	Stretching Vibration C=C
1377	1380	Stretching Vibrations CH_3
1267	1267	Stretching C-O ester
1126	1124	Stretching vibration C-O C
1076	1070	Stretching primary alcohol OH
-	1033	Stretching primary alcohol OH
-	960	Trans CH wag
875	890	Out-of-plane angular deformation OH (carboxilic ácid)
740	736	Out-of-plane bending C-H (alkene)
-	700	Out-of-plane bending C=C or NH out-of plane bending

3.2 Volatiles production

Several volatile compounds were detected in the extract of the fermented orange waste by *Phomopsis* sp. Among then, (R)-(-)-carvone, α-terpineol, 1,6-dihydrocarveol, (-)-trans-isopiperitenol, (1S,2S,4R)-(+)-limonene-1,2-diol and α-limonene diepoxide are the most noticeable among the compounds identified via GC-MS analysis (Table 4). These compounds are most important because they are derivatives of limonene (Maróstica Júnior and Pastore, 2007). The major differences between the extracts of orange waste and the fermented orange waste is the presence of a high number of mono-, di- and triterpenoids in the precursor waste such as D-verbenone, 3-carene, β-cadinene, azulene, germacrene and naphthalene whereas in the fermented orange waste the

production of more complex derivatives such as limonene-1,2-diol, cis-linalool oxide, limonene-diepoxide, trans-isopulegone and the main fermentation products. Also, in the precursor waste extract, the high amount of esters and carboxylic acids (n-butyl acetate, propanoic acid, mirystic acid) is observed while the fermented waste extract more alcohols (caprilic alcohol, dihydrocarveol, limonene-1,2-diol and others) and some interesting compounds like valencene, sabinene and α-tocopherol, which have some known important bioactive properties (Liu *et a.,* 2012; Valente e*t al.* 2013; Madhavi *et al.* 1996).

Table 4: Major volatile compounds identified by gas chromatography-mass spectrometry (GC- MS) in the orange waste extract and fermented orange waste by *Phomopsis* sp.

Compound	CAS	OWE[1]	FE[2]	MS Fragments
n-Butyl acetate	123-86-4	97%	- [3]	43,56,87,115
α-Pinene	80-56-8	95%	88%	39, 53, 93, 107, 136, 154
β-Phellandrene	555-10-2	90%	-	41, 65, 93, 121, 136
(+)-Sabinene	3387-41-5	-	94%	41, 77, 93, 121, 136
β-Myrcene	123-35-3	95%	95%	41, 69, 93, 121, 136
β-Pinene	127-91-3	96%	90%	41, 69, 93, 121, 136
3-Carene	13466-78-9	96%	-	41, 67, 93, 121, 136
Cis-β-Ocimene	3338-55-4	-	92%	41, 77, 93, 105, 136
(R)-(+)-Limonene	5989-27-5	95%	95%	41, 68, 93, 121, 136
Caprylic alcohol	111-87-5	-	95%	41, 56, 84, 112, 129
β-Phenylethyl alcohol	60-12-8	-	97%	39, 65, 91, 122
(-)-Linalool	78-70-6	94%	92%	41, 71, 93, 121, 136, 154
Trans-p-mentha-2,8-dien-1-ol	7212-49-0	92%	-	43, 67, 79, 109, 134, 152
Cis-p-mentha-2,8-dien-1-ol	22771-44-4	92%	91%	43, 67, 91, 109, 137, 152
Trans-isopulegone	29606-79-9	-	91%	41, 67, 93, 109, 134, 152
(-)-Trans-isopiperitenol	74410-00-7	-	94%	41, 69, 84, 108, 134, 152
α-Terpineol	98-55-5	94%	91%	43, 59 93, 95, 121, 139
D-Verbenone	18309-32-5	83%	-	39, 55, 91, 107, 135, 150
Cyclohexyl isothiocyanate	1122-82-3	86%	-	41, 55, 83, 109, 141
Trans-Carveol	1197-07-5	-	84%	41, 55, 84, 109, 134. 152
Cis-Carveol	1197-06-4	-	87%	41, 55, 84, 109, 134. 152
Neodihydrocarveol	18675-34-8	-	88%	41, 55, 93 107, 136, 154
1,6-Dihydrocarveol	619-01-2	-	87%	41, 68, 93, 107, 136, 154
α-Limonene-dipoxide	96-08-2	-	88%	43, 67, 79,107, 123. 153, 168
D-Carvone	2244-16-8	92%	-	39, 54, 82, 108, 135, 150

(R)-(-)-Carvone	6485-40-1	-	89%	39, 54, 82, 109, 135, 150
Cis-linalool oxide	1365-19-1	-	86%	43, 59, 94, 111, 137, 155
Trans-ascaridol glycol	21473-37-0	-	86%	43, 55, 81, 109, 127, 152
(1S,2S,4R)-(+)-Limonene-1,2-diol	38630-75-0	-	96%	43, 71, 82, 108, 137, 152
γ-Muurolene	30021-74-0	88%	-	41, 67, 91, 119, 133, 161, 189, 204
α-Copaene	3856-25-5	97%	-	41, 55, 91, 119, 133, 161, 189, 204
β-Copaene	18252-44-3	91%	-	41, 55, 91, 105, 133, 161, 189, 204
Caryophyllene	87-44-5	96%	-	41, 69, 93, 105, 133, 161, 189, 204
Humulene	6753-98-6	92%	-	41, 55, 93, 121, 147, 161, 189, 204
D-Germacrene	23986-74-5	94%	-	41, 67, 91, 105, 133, 161, 189, 204
Valencene	4630-07-3	-	92%	41, 67, 91, 105, 133, 161, 189, 204
Naphthalene	91-20-3	94%	-	41, 67, 91, 105, 133, 161, 204
α-Muurolene	31983-22-9	94%	-	41, 77, 93, 105, 133, 161, 189, 204
Azulene	275-51-4	91%	-	41, 55, 93, 107, 135, 161, 189, 204
Butylated hydroxytoluene	128-37-0	86%	-	41, 57, 91, 105, 145, 161, 177, 205
β-Cadinene	523-47-7	90%	-	41, 55, 81, 119, 134, 161, 189, 204
Caryophyllene oxide	1139-30-6	88%	-	43, 69, 79, 109, 135, 149, 177, 220
Trimethylsilyl ester of tetradecanoic acid	18603-17-3	92%	-	43, 73, 83, 117, 129, 159, 185, 201, 241, 257, 285, 300
Methyl Palmitate	112-39-0	95%	-	43, 74, 87, 115, 143, 171, 185, 199, 227, 256, 270
Dioctyl isophthalate	117-81-7	-	92%	41, 70, 83, 112, 149, 167, 261, 279
L-Ascorbic acid	50-81-7	89%	89%	43, 57, 85, 115, 129, 157, 185, 213, 239, 256,
Ethyl Palmitate	628-97-7	92%	91%	43, 55, 88, 115, 143, 157, 199, 239, 284
Linoleic Acid	60-33-3	94%	-	43, 61, 81, 109, 136, 150, 178, 220, 263, 279, 294
Methyl octodeca-9,12-dienoate	2462-85-3	-	90%	41, 67, 81, 109, 136, 150, 178, 263. 294
Citric acid	77-92-9	93%	-	43, 57, 87, 112, 129, 157, 185, 213, 231, 259, 273, 305, 329, 343
α-Tocopherol	10191-41-0	-	88%	43, 57, 91, 121, 136, 165, 205, 430
Eicosane	112-95-8	94%	-	43, 50, 85, 113, 127, 155, 183, 211, 225, 253, 282

*F.E: extract of a fermented orange waste; O.E: orange waste extract. - Means no detection.

The presence of carboxylic acids, alcohols, carboxylic esters, terpenoids and hydrocarbons corroborate with the results obtained in the infrared analysis. However, no nitrogen compounds were found in the GC-MS analysis, except for cyclohexyl isothiocyanate.

The limonene biotransformation was performed during seven days in order to find the best time for the production of limonene derivatives (figure 2). The highest

concentration of limonene-1,2-diol was achieved after 144 h of fermentation (2.66 g/kg substrate). Limonene-1,2-diol or limonene-glycol is a colorless to very slightly yellow oil with a cool minty aroma during consumption, and having the odor and/or flavor used in mint (Burdock,2010). Others compounds significantly produced were α-terpineol (0.44 g/kg of substrate), trans-carveol (0.13 g/kg of substrate) and cis-carveol (0.21 g/kg of substrate).

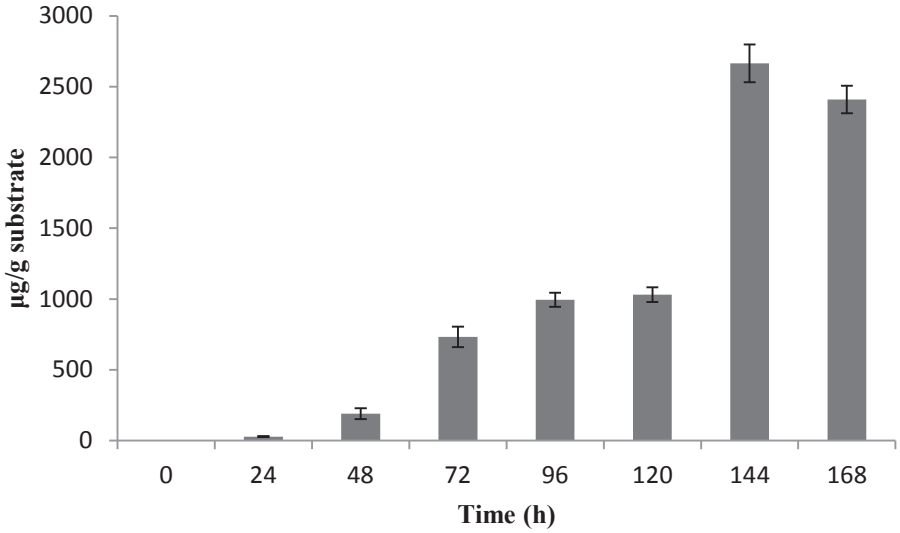


Figure 2 – Limonene-1,2-diol concentration during 168 h of solid state fermentation of orange waste by *Phomopsis* sp.

It is important to notice that 144 h (the maximum production) may be considered as a long fermentation period regarding the limonene biotransformation process. The product was, howerver, already present after 24 h of fermentation, as ocurred with Bicas *et al.* (2008), who reported the production of α-terpineol and limonene-1,2-diol after 24 hours of fermentation by *Pseudomonas*, while *Penicillium digitatum* was able to convert limonene into (R)-(+)-α-terpineol after only 8 h (Adams *et al.* 2003). In spite of this, Demyttenaere et al (2001a) described the bioconversion to limonene-1,2-diol as a slow process as it occurred at the end of the experiment with 5 days. Rottava *et al.,* (2011) achieved good yields for the production of α-terpineol after 144 h of fermentation with an isolated yeast. The first two studies were performed, however, in submerged fermentation at low concentrations of limonene, while the concentration of the limonene in the orange waste media with 80% of umidity is higher than 1%. As regarding

endophytes, Molina *et al.* (2013) achieved the maximum concentration of verbenol from β-pinene after 85 h of fermentation and the production started after 48 h. In our study, significant concentrations of limonene-1,2-diol were firstly achieved after 48 h (191.60 µg/g substrate) and 72 h (732.96 µg/g substrate) of fermentation. Few studies have been reported with biotransformation experiments regarding endophytes, hence, some of its fermentation features are still unknown. However, it presents similar fermentation times to some filamentous fungi and is usually different from bacteria as stated before. Also, the behavior cannot be fully compared once the biotransformations already reported occur in submerged fermentation. fermentation.

After a factorial design 3^{3-1} including pH, particle size and inoculum volume, the concentration of α-terpineol increased from 0.44 g/kg to 0.72 g/kg when the granulometry was at 2-3 mm, pH 6.0 and inoculum size of 3 mL. The production of α-terpineol has already been reported at higher concentrations by Bicas *et al.* (2008) – 2.4 g/L and Tan *et al.* (1998) – 3.2 g/L, both in submerged fermentation in a synthetic medium by fungi. The highest production of limonene-1,2-diol also increased and occurred at pH 6.0 with a particle size of 0.8-2.0, and reached 3.02 g/kg of substrate after 144 h of fermentation.

An achievement of 3.02 g/kg of substrate is superior to most reports of limonene-1,2-diol, which is usually the minor fermentation product (Molina and Pastore, 2013; Demyttenaere *et al.* 2001b; Carvalho *et al.* 2003) but also superior to the reports of it as a major fermentation product, such as by Mukherjee *et al.* (1973) using *Cladosporium* sp. (1.5 g/L). However, this study presents, a lower concentration than Molina *et al.* (2015) - 3.7 g/L of limonene-1,2-diol using S(-) limonene as substrate in a synthetic medium for a filamentous fungus. Yes, high concentrations of limonene-1,2-diol has only been achieved with the use of endophytes by *Bier et al.,* (2017) and also, no production of α-terpineol or limonene-1-2-diol had been reported on solid state fermentation.

3.3 Antioxidant activity by DPPH

The antioxidant capacity of the fermented extract was established by the comparison of its activity to butylated hydroxyanisole (BHA) and ascorbic acid. Figure 3 shows the antioxidant activity of the fermented extract in comparison to the blank.

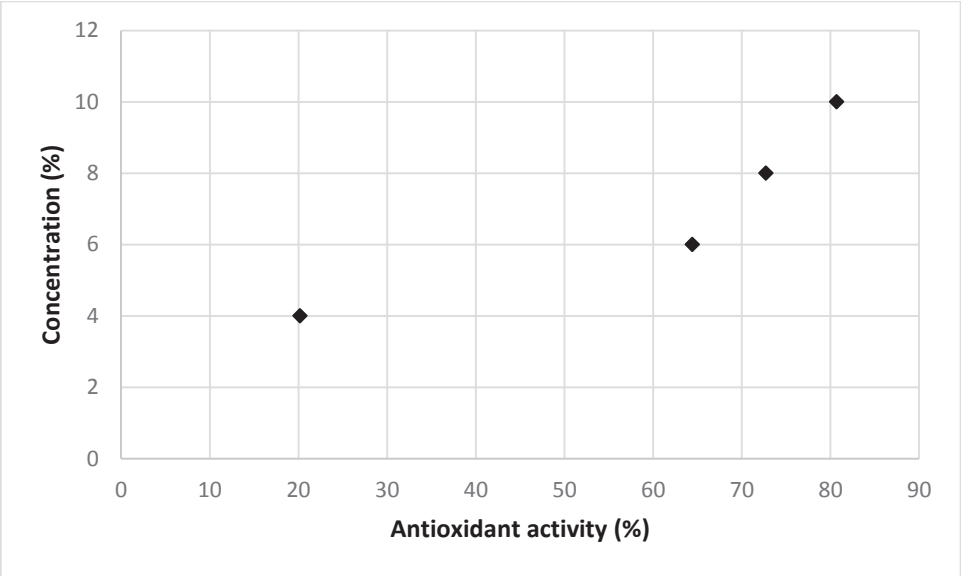


Figure 3: Antioxidant activity of the fermentated extract (in comparison to the blank in the same concentrations).

The orange waste extract (diluted 1:10) at a concentration of 5.79% has the antioxidant equivalent capacity to 85.938 mMol of BHA or 107.277 mMol of ascorbic acid. Furthermore, the IC 50 is 5.79% of the original extract.

The fermented orange waste extract was able to reduce the stable, purple-colored radical DPPH, considering the 80.68% inhibition with an initial concentration of 10% (1:10). At 4%, the fermented orange waste extract presented 20.17% inhibition, while the orange waste extract (peel and bagasse) had 12.11% inhibition at the same concentration. Sarrou *et al.* (2013) determined the scavenging activity of flowers, young leaves and peel oil of citrus as 53.98%, 22.79% and 19.29%, respectively, while the antioxidant potential of the essential oil of two specimen of *Citrus sinensis* was 10.5% and 30% (Malhotra *et al.* 2009). At last, the antioxidant potential of the fermented orange waste extracts by *Phomopsis sp.* reached significant results, principally if compared to the orange oil results. It proves that the compounds produced by the fungus presented a higher antioxidant activity than their precursors.

The DPPH results of limonene and limonene-1,2-diol showed that the latter compound had higher antioxidant capacity (5.67% ± 0.89) than limonene (3.65% ± 0.72) at a concentration of 4% (v/v). This result demonstrates that the production of limonene-1,2-diol increases the antioxidant capacity of the extract as it has higher potential than its precursor, limonene. However, not only the fermentation extract, but also the orange waste showed higher antioxidant capacity than the standards. Therefore, the antioxidant activity of the extracts may be attributed to other mono-, di- and sesquiterpenes present in the orange waste extract and their derivatives present on the fermented extract.

3.4 Antioxidant capacity by CUPRAC

The antioxidant activity by the Cuprac method indicated that the fermented orange waste extract has the highest antioxidant activity (538.05 mg TE per g of dry extract) (figure 4). When compared to the orange residue extract (168.27 mg TE per g of dry extract) it has 3.2 times more antioxidant activity ($p<0.01$). The results of the orange residue extract are in accordance with the results obtained by Assefa *et al.* (2016) that verified the activity of different extracts of citrus fruits ranging from 16.8 to 208.7 mg TE per g of dry extract. The antioxidant potential shown by the fermentation extract is also highly significant when compared to the studies of Stojković *et al.* (2014) that obtained extracts from *Tanacetum vulgare* with different solvents from the leaves and flowers ranging from 33 to 214 mg TE per g of dry extract and Ordoudi *et al.* (2014), who reported 84.71 mg trolox per g of dry weight of pomegranate juice.

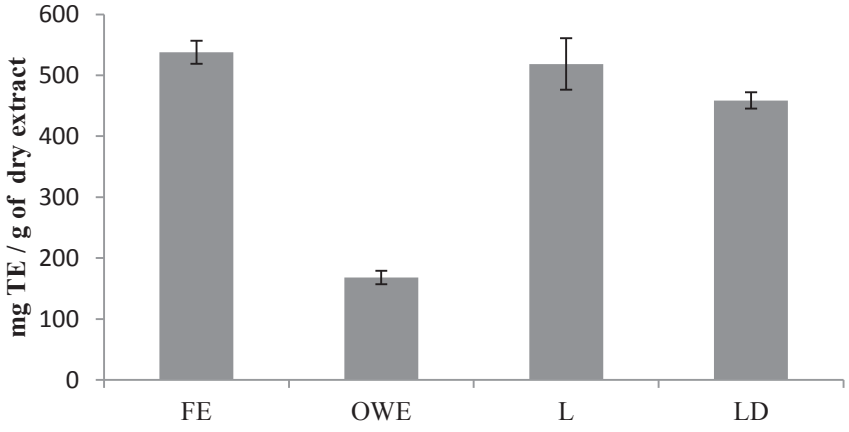


Figure 4: Antioxidant activity of the standards and the extracts by CUPRAC (mg TE / g dry extract). FE: Fermented orange waste extract, OWE: Orange waste extract, L: Limonene and LD: Limonene-1,2-diol.

The results obtained with limonene (519 mg TE / g) are slightly higher than those obtained with limonene-1,2-diol (459 mg TE / g), but this is not significant statistically ($p>0.5$). The results obtained with the standards are similar to the results of the fermentation extract ($p>0.1$). They indicate that the increase of the antioxidant potential of the fermentation extract does not occur due to the bioconversion of limonene in limonene-1,2-diol, but because of the variety of compounds with related antioxidant activity on the fermentation extract. The low antioxidant activity of the orange waste extract is quite visible, despite its high content of limonene. It can be concluded that the compounds in its composition such as monoterpenes and esters may have contributed little to the antioxidant capacity.

3.5 Antioxidant capacity by ORAC

The antioxidant activity by the ORAC method (table 5) indicates an increase in the antioxidant activity of the fermented orange waste extract (24011.39 ± 640.16 μmolTE/g) in relation to the natural potential of the orange waste extract (5226.45 ± 23.04 μmolTE/g). The results obtained for the antioxidant capacity of the waste constituted of orange peel and bagasse is in accordance with the results obtained by Khan *et al.* (2010), who tested extraction methods on citrus fruits that ranged from 5009 to 7120 μmolTE/g. The orange waste extract activity with the ORAC method was higher than the values described by Jayaprakasha *et al.* (2008) with citrus fruits (2220.72 ± 22 μmolTE/g). This superior result is explained by the efficiency of the extraction method used. It is the almost three times increase of the antioxidant capacity of the fermentation extract in this method, reaching an ORAC value similar to that obtained by jabuticaba peel oil (Parashar *et al.* 2014), is important to note.

Table 5: Antioxiant capacity of the extracts and standards by the ORAC method

Compound / material	**Antioxidant capacity (**μmolTE/ g)
Orange waste extract	5226.45± 23.04
Fermented orange waste extract	24011.39± 640.16
Limonene	5262.87± 33.0
Limonene-1,2-diol	18220.89 ±1080.88

In this method, there was a high correlation between the antioxidant capacity of limonene and the orange waste extract, the fermentation extract and limonene-1,2-diol

and the production of limonene-1,2-diol from limonene. Limonene showed a potential of 5262.87 μmolTE/g, while limonene-1,2-diol had and ORAC value of 18220.89 μmolTE/g.

The results obtained by the ORAC method are in accordance with the results obtained by CUPRAC. The increase of the antioxidant activity of the fermented orange waste in comparison to the orange waste extract is similar for both methods. However, in the CUPRAC method, there was not a direct relation between the production of limonene-1,2-diol and the antioxidant capacity of the fermented orange waste extract, due to the similar antioxidant capacity shown by limonene and limonene-1,2-diol in that method.

3.6 Total polyphenols content

The results obtained with the Folin-Ciocalteu reagent show an increase of more than 8 times of the polyphenols content from the original orange extract (36.39 ± 1,97 mg galic acid/g extract) in comparison to the fermentated extract (271.33 ± 3,73 mg/g). The results obtained from the orange waste are a bit higher than the obtained by Ma *et al.* (2008), which reached 19.12 ± 0.21 mg/g, Furthermore, the polyphenols content present here a direct correlation with the antioxidant activity obtained with Cuprac, Orac and DPPH methods, that showed an improvement of the antioxidant capacity of the fermentation extract.

4. Conclusion

This study presents new perspectives in a solid state fermentation process on the use of endophytes for a biotechnological process, wiling a natural waste mediums. Limonene-1-2-diol and α-terpineol were produced by conversion of limonene orange waste, opening the potential for further studies regarding the use of solid state fermentation for the biotransformation of limonene and possibly other terpenoids. It also confirms the crescent importance of the endophyte fungi in various biotechnological aspects.

The results obtained in all of the antioxidant tests using the fermented orange waste extract were very positive. There was a substantial increase in the antioxidant activity of the orange oil after the fermentation process in all of the four methods used. The lack of

information about the properties of limonene-1,2-diol is still a problem for the production and use of this limonene derivative in the flavor industry. This paper thus achieves a series of new statements regarding the biotechnological biotransformation of limonene and offers new perspectives to it showing a great prospect in the potential of the fermented extract of *Phomopsis* sp. in the antioxidant activity.

References

Adams, A., Demyttenaere J.C.R. and De Kimpe N. (2003) Biotransformation of (R)-(+)- and (S)-(-)-limonene to α-terpineol by *Penicillium digitatum*— investigation of the culture conditions. Food Chemistry **80**, 525-534.

Akacha, N.B. and Gargouri, M. (2015) Microbial and enzymatic technologies used for the production of natural aroma compounds: Synthesis, recovery modeling, and bioprocesses. Food and Bioproducts Processing **94**, 675–706.

Apak, R., Güçlü, K., Demirata, B., Özyürek, M., Çelik, S.E., Bektaşoğlu, B., Berker, I. and Özyurt, D. (2007) Comparative evaluation of various total antioxidant capacity assays applied to phenolic compounds with the CUPRAC assay. Molecules **12**, 1496-1547.

Arce, A., Pobudkowskal, A. Rodríguez, O. and Soto, O. (2007) Citrus essential oil terpenless by extraction using 1-ethyl-3-methylimidazolium ethylsulfate ionic liquid: Effect of the temperature. Chemical Engineering Journal **133**, 213-218.

Assefa, A.D. Ko, E.Y., Moon, S.H. and Keum, Y-S. (2016) Antioxidant and antiplatelet activities of flavonoid-rich fractions of three citrus fruits from Korea. 3 Biotech **6**, 1-10.

Bacanli, M., Basaran, A.A. and Basaran, N. (2015) The antioxidant and antigenotoxic properties of citrus phenolics limonene and naringin. Food Chemical Technology **81**, 160-170

Badee, A.Z.M., Helmy, S. A. and Morsy, N. F. S. (2011) Utilisation of orange peel in the production of α-terpineol by *Penicillium digitatum* (NRRL 1202). Food Chemistry **126**, 849–854.

Barbosa, L.C.A. (2007) Espectroscopia no Infravermelho na caracerização de Compostos Orgânicos. Editora UFV, 1st , 2007, 189 p.

Bicas, J.L., Fontanille, P., Pastore, G.M. and Larroche, C. (2008) Characterization of monoterpene biotransformation in two pseudomonads. Journal of Applied Microbiology **105**, 1991-2001.

Bier, M.C.J., Poletto, S., Soccol, V. T., Soccol, C. R., Medeiros, A. B. P. (2011) Isolation and Screening of Microorganisms with Potential for Biotransformation of Terpenic Substrates. Brazilian Archives of Biology and Technology **54**, 1019-1026.

Bier, M.C.J., Medeiros, A.B.P., De Oliveira, J.S., Côcco, L.C., Costa, J.L., Soccol, C. R. (2016) Liquefied gas extraction: A new method for the recovery of terpenoids from agroindustrial and forest wastes. The Journal of Supercritical Fluids **110**, 97-102.

Bier, M.C.J., Medeiros, A.B.P., Soccol, C. R. (2017) Biotransformation of limonene by an endophytic fungus using synthetic and orange residue-based media. Fungal Biology **121**, 137-144.

Burdock, G. A. (2010) Fenaroli's Handbook of Flavor Ingredients, 6 ed. Boca Raton: CRC Press.

Carvalho, C.C.R. and Fonseca, M.M.R. da. (2003) Towards the bio-production of trans-carveol and carvone form limonene: induction after cell growth on limonene and toluene. Tetrahedron: Asymmetry **14**,3925-3931.

Carvalho, C.C.R. and Fonseca, M.M.R. da. (2006) Biotransformation of terpenes. Biotechnology Advances **24**, 134–142.

Chen, Z., Chu, H., Chyau, C., Chu, C. and Duh, P. (2012) Protective effects of sweet orange (Citrus sinensis) peel and their bioactive compounds on oxidative stress. Food Chemistry **135**, 2119–2127.

Crizel, A. Jablonski, A.O., Rios, R., Rech, S.H. and Flôres, S. H. (2013) Dietary fiber from orange byproducts as a potential fat replacer. Food Science and Technology **53**, 9–14.

Das, R., K. Brar, S. K. and Verma, M. (2016) Potential use of pulp and paper solid waste for the bio-production of fumaric acid through submerged and solid state fermentation. Journal of Cleaner Production **112**, 4435-4444.

Demyttenaere, J.C.R., Belleghem, K.V., De Kimpe, N. (2001a) Biotransformation of (R)-(+)- and (S)-(-)-limonene by fungi and the use of solid phase microextraction for screening, Phytochemistry **57**, 199-208

Demyttenaere, J.C.R., Belleghem, K.V., De Kimpe, N. (2001b) Biotransformation of terpenes by fungi: Study of the pathways involved, Journal of Molecular Catalysis B: Enzymatic **11**, 265–270.

Diaz, S., Espinosa, S., and Brignole, E.A. (2005) Citrus peel oil deterpenation with supercritical fluids optimal process and solvent cycle design. Journal of Supercritical Fluids **35**, 49-61.

El-Akhal, F., Lalami, A.E. and O. Guemmouh, R. (2015) Larvicidal activity of essential oils of Citrus sinensis and Citrus aurantium (Rutaceae) cultivated in Morocco against the malaria vector *Anopheles labranchiae* (Diptera: Culicidae). Asian Pacific Journal of Tropical Disease **5**, 458-462.

Fiorentin, L.D., Menon, B.T., de Barros, S.T.D., Pereira, N.C., Lima, O.C.M. and, Modenes, A.N. (2010) Isotermas de sorção do resíduo agroindustrial bagaço de laranja. Revista Brasileira de Engenharia Agrícola e Ambiental **14**, 653–659.

Fisher, K. and Phillips, C. (2008) Potential antimicrobial uses of essential oils in food: is citrus the answer?. Trends in Food Science & Technology **9**, 156-164.

Gosslau A., Chen, K. Y., Ho, C. and Li, S. (2014) Anti-inflammatory effects of characterized orange peel extracts enriched with bioactive polymethoxyflavones. Food Science and Human Wellness **3**, 26-35.

Huang, D., Ou, B., Hampsch- Woodill, M., Flanagan, J., and Prior, R. (2002) High-throughput Assay of Oxygen Radical Absorbance Capacity (ORAC) Using a Multichannel Liquid Handling System Coupled with a Microplate Fluorescence Reader in 96-Well Format. Journal of Agriculture and Food Chemistry **50**, 4437-4444.

Hue, C., Gunata, Z., Bergounhou, A., Assemat, S., Boulanger, R., Sauvage, F.X. and Davrieux, F. (2014) Near infrared spectroscopy as a new tool to determine cocoa fermentation levels through ammonia nitrogen quantification. Food Chemistry **148**, 240-245.

Instituto Adolfo Lutz, Normas Analíticas do Instituto Adolfo Lutz (Vol 1: Métodos Físico-Químicos para Análise de Alimentos), 4th ed., São Paulo, 2008, p.117.

Jayaprakasha, G.K., Girennavar, B. and Pati, B.S. (2008) Antioxidant capacity of pummelo and navel oranges: Extraction efficiency of solvents in sequence. LWT – Food Science and Technlogy **41**, 376-384.

Kaur, J. and Kaur, G. (2015) An insight into the role of citrus bioactives in modulation of colon cancer. Journal of Functional Foods 13 (2015) 239-261.

Liu, K., Chen, Q., Liu, Y., Zhou, X. and Wang, X. (2012) Isolation and biological activities of decanal, linalool. valencene, and octanal from sweet orange oil, Journal of Food Science **77**, 1156-1161.

Lu, Y., Zhao, S., Zhang, J., and Zhang, H. (2012) Xie, Z. Cai, G. Jiang, W. Effect of orange peel essential oil on oxidative stress in AOM animals. International Journal of Biological Macromolecules **50**, 1144-1150.

Ma, Y-Q, Chen, J-C, Liu, D-H and Ye, X-Q. (2008) Effect of Ultrasonic Treatment on the Total Phenolic and Antioxidant Activity of Extracts from Citrus Peel. Journal of Food Science **73**, 115-120.

Madhavi, D.L., Deshpande, S.S. and Salunkhe, D. K. (1996) Food Antioxidants: Technological, Toxicological and Health Perspectives. New York: Marcel Dekker.

Mahmood, A.U., Greenman, J. and Scragg, A.H. (1998) Orange and potato peel extracts: Analysis and use as *Bacillus* substrates for the production of extracellular enzymes in continuous culture. Enzyme and Microbial Technology **22**, 130-137.

Malhotra, S., Suri, S. and Tuli, R. (2009) Antioxidant activity of citrus cultivars and chemical composition of *Citrus karna* essential oil. Planta Médica **75**, 62-44.

Mantzouridou, F.T., Paraskevopoulou, A. and Lalou, S. (2015) Yeast flavour production by solid state fermentation of orange peel waste. Biochemical Engineering Journal **101**, 1–8.

Maróstica Jr, M.R. and Pastore, G. M. (2007) Production of *R*-(+)-α-Terpineol by the biotransformation of limonene from orange essential oil, using cassava waste water as medium. Food Chemistry 101, 345-350.

Maróstica Júnior, M.R. and Pastore, G. M. (2007) Biotransformation of limonene: a review of the main metabolic pathways. Química Nova **30**, 382-387.

Molina, G., Bution, M.L., Bicas, J.L., Dolder, M.A.H. and Pastore, G.M. (2015) Comparative study of the bioconversion process using R-(+)- and S-(–)-limonene as substrates for *Fusarium oxysporum* 152B. Food Chemistry **174**, 606–613.

Molina, G. and Pastore, G.M. (2013) Biotransformation of Monoterpenes by Endophytes Isolated From Brazilian Fruits. International Proceedings of Chemical, Biological & Environmental **50**, 59-64.

Molina, G., Pimentel, M.R., Bertucci, T.C.P. and Pastore, G.M. (2012) Application of fungal endophytes in biotechnological processes. Chemical Engineering Transactions. **27,** 289-294.

Mukherjee, B.B., Kraidman, G. and Hill, I. D. (1973) Synthesis of Glycols by Microbial Transformation of Some Monocyclic Terpenes. Applied Microbiology **25**, 447-453.

Murthy, K.N.C., Jayaprakasha, G. K. and Patil, B. S. (2012) D-limonene rich volatile oil from blood oranges inhibits angiogenesis, metastasis and cell death in human colon cancer cell. Life Sciences **91**, 429-439.

Ngutaa, J.M., Mbaria, J.M., Gakuya, D.W., Gathumbi, P.K., Kabasa, J. D. and Kiama, S. G. (2012) Evaluation of Acute Toxicity of Crude Plant Extracts from Kenyan Biodiversity, using Brine Shrimp, *Artemia salina* L. (Artemiidae). The Open Conference Proceedings Journal **3**, 30-34

Nicolini, L., Hunolstein, C.V. and Carilli, A. (1987) Solid state fermentation of orange peel and grape stalks by *Pleurotus ostreatus*, *Agrocybe aegerita*, and *Armillariella mellea,* Applied Microbiology and Biotechnology **26**, 95-98.

NIST 14 Mass Spectral Library & Search Software v.2.2 (2014-04) (NIST 2014/EPA/NIH) (2014) National Institute of Standards and Technology, Gaithersburg Md, USA.

Oliveira, J. S. (2000) Extrator para Operação com Gases Liquefeitos, Brazilian Patent PI 9804199-1.

Ordoudi, S. A., Mantzouridou, F., Daftsiou, E., Malo, C., Hatzidimitriou, E., Nenadis, N. and Tsimidou, M. Z. (2014) Pomegranate juice functional constituents after alcoholic and acetic acid fermentation. Journal of Functional Foods **8**, 161-168.

Pandey, A. (2003) Solid state fermentation. Biochemical Engineering Journal **13**, 81–88.

Pandey, A., Soccol, C.R. and Larroche, C. (2008) Production of aroma compounds**.** In: Current developments in Solid-state Fermentation. Asiatech publishers Inc, India: Springer.

Parashar, S., Sharma, H. and Garg, M. (2014) Antimicrobial and Antioxidant activities of fruits and vegetable peels: A review. Journal of Pharmacognosy and Phytochemistry **3**, 160-164

Pourbafrani, M., Forgács, G., Horváth, I. S., Niklasson, C. and Taherzadeh M. J. (2010) Production of biofuels, limonene and pectin from citrus wastes. Bioresource Technology **101**, 4246-4250.

Qadri, M., Deshidi, R., Shah, B.A., Bindu, K., Vishwakarma, R.A. and Riyaz-Ul-Hassan, S. (2015) An endophyte of *Picrorhiza kurroa* Royle ex. Benth, producing menthol, phenylethyl alcohol and 3-hydroxypropionic acid, and other volatile organic compounds. World Journal of Microbiology and Biotechnology **31**, 1647-1654.

Reda, S.Y., Leal, E.S., Batista, A.C., Barana, A.C., Schinitzel, E. S. and Carneiros, P.I.B. (2005) Caracterização dos Óleos das Sementes de Limão Rosa (*Citrus limonia* Osbeck) e limão siciliano (*Citrus limon*), um Resíduo Agroindustrial. Ciência e Tecnologia de Alimentos **25**, 672-676.

Rottava, I., Cortina, P.F., Martello, E., Cansian, R.L., Toniazzo, G., Antunes, O.A., Oestreicher, E.G., Treichel, H. and de Oliveira, D. (2011) Optimization of α-Terpineol production by the biotransformation of R-(+)-limonene and (-)-β-pinene. Applied Biochemistry and Biotechnology **164**, 514-523.

Santi, G., Crognale, S., D'Annibale, A., Petruccioli, M., Ruzzi, M., Valentini, R. and Moresi M. (2014) Orange peel pretreatment in a novel lab-scale direct steam-injection apparatus for ethanol production. Biomass and Bioenergy **61**, 146–156.

Sarrou, E., Chatzopoulou, P., Dimassi-Theriou, K. and Therios, L. (2013) Volatile Constituents and Antioxidant Activity of Peel, Flowers and Leaf Oils of *Citrus aurantium* L. Growing in Greece. Molecules **18**, 10639-10647.

Schmidt, C.G., Furlong, E.B. (2012) Effect of particle size and ammonium sulfate concentration on rice bran fermentation with the fungus *Rhizopus oryzae*. Bioresource Technology **123**, 36–41.

Silva, A.L.L. Rodrigues. C. Costa, J.L. Machado, M.P. Penha, R.O. Biasi, L.A. Vandenberghe, L.P.S. Soccol, C.R. (2013) Gibberellic acid fermented extract obtained

by solid-state fermentation using citric pulp by *Fusarium moniliforme*: Influence on *Lavandula angustifolia* Mill., cultivated *in vitro*. Pakistan Journal of Botany **45**, 2057-2064.

Silvestein, R.M., Bassler, G.C., and Morril, T.C. (1991) Spectroscopic identification of organic compounds. 5th ed. John Wiley & Sons. 419 p.

Singleton, V.L. and Rossi, J.A. Jr. (1965) Colorimetry of total phenolics with phospomolybdic–phospotungstic acid reagents. American Journal of Enology and Viticulture **16,** 144-158.

Smit, A.Y. Engelbrecht, L. Toit, M. (2012) Managing Your Wine Fermentation to Reduce the Risk of Biogenic Amine Formation. Frontiers in Microbiology **3**, 1-10.

Soccol, C.R., Medeiros, A.B.P., Bier, M.C.J. Bioprocesso para a produção de derivados de terpenos por *Diaporthe sp.* e *Rhodotorula mucilaginosa.* Patente INPI protocolo BR 102014029813, 28/11/2014.

Song, F-L., Gan, R-Y., Zhang, Y., Xiao, Q., Kuang, L. and Li, H-B. (2010) Total Phenolic Contents and Antioxidant Capacities of Selected Chinese Medicinal Plants, International Journal of Molecular Sciences **11**, 2362-2372.

Stojković, M.B., Mitić, S.S., Pavlović, J.L., Stojanović, B.T. and Paunović, D.D. (2014) Antioxidant potential of *Tanacetum vulgare* L. extracts. Biologica Nyssana **5**, 47-51.

Tan, Q., Day, D.F. and Cadwallader, K.R. (1998) Bioconversion of (R)-(+)-limonene by *P. digitatum* (NRRL 1202). Process Biochemistry **33**, 29-31.

United States Department of Agriculture. (2017) Citrus: World Markets and Trade. Available in: <https://apps.fas.usda.gov/psdonline/circulars/citrus.pdf>, Accessed in: 09/03/2017.

Valente, J., Zuzarte, M., Gonçalves, M.J., Lopes, M.C., Cavaleiro, C., Salgueiro, L. and Cruz, M.T. (2013) Antifungal, antioxidant and anti-inflammatory activities of *Oenanthe crocata* L. essential oil. Food and Chemical Toxicology **62**, 349-354.

Vanek, T., Valterová, I., Vanková, R. and Vaisar, T. (1999) Biotransformation of (-)-limonene using *Solanum aviculare* and *Dioscorea deltoidea* immobilized plant cells. Biotechnology Letters **21**, 625-628.

Velázquez-Nuñez, M.J., Avila-Sosa, R., Palou, E. and López-Malo, A. (2013) Antifungal activity of orange (*Citrus sinensis* var. Valencia) peel essential oil applied by direct addition or vapor contact. Food Control **31**, 1-4.

Wang, Y. and Dai, C-C. (2011) Endophytes: a potential resource for biosynthesis, biotransformation, and biodegradation. Annals of Microbiology **61**, 207-215.

Yadava, A.R., Chauhan, A.S., Rekha, M.N. and Ramteke, R.S. (2004) Flavour quality of dehydrated lime [*Citrus aurantifolia* (Christm.) Swingle]. Food Chemistry **85**, 59-62.

Yang, F-C., Ma, T-W. and Lee, Y-H. (2013) Reuse of citrus peel to enhance the formation of bioactive metabolite-triterpenoid in solid-state fermentation of *A. cinnamomea*. Biochemical Engineering Journal **78**, 59–66.

Zulueta, A., Esteve, M.J. and Frígola, A. (2009) ORAC and TEAC assays comparison to measure the antioxidant capacity of food products. Food Chemistry **114**, 310-316.

Capítulo 5: Biotransformation of orange waste by *Diaporthe* sp. and evaluation of the immunomodulatory and antitumoral activity of the fermented product

Mário César Jucoski Bier[1], Adriane Bianchi Pedroni Medeiros[1*], Suzany Hellen da Silva[2], Rosália Rubel[2], Sandro José Ribeiro Bonatto[2], and Carlos Ricardo Soccol[1]

[1] Department of Bioprocess Engineering and Biotechnology, Universidade Federal do Paraná, R. Francisco H. dos Santos, CEP 81531-990, Curitiba, PR, Brazil

[2] Instituto de Pesquisa Pelé Pequeno Príncipe, Av. Silva Jardim, 1632 , CEP 80250-060, Curitiba, PR, Brazil.

* Corresponding Author: Adriane Bianchi Pedroni Medeiros

Department of Bioprocess Engineering and Biotechnology, Universidade Federal do Paraná

81531-970, Curitiba PR, Brazil

E-mail: adrianebpm@ufpr.br

Tel.: +55 41 33613697

Fax: +55 41 33613695

ABSTRACT

The study of bioactivities of essential oils has been highly explored recently. Limonene, the main compound of orange essential oil, and its derivatives already have a number of reported bioactive properties, such as antioxidant and antitumoral activity. While most of the studies focus on the bioactive potential of fruits or chemical standards, few articles have tested the potential of the fermented products or successfully verified the potential of the endophytic fungus in this area. The main objective of this study was to evaluate the *in vitro* abilities of immunomodulation, which is pretty scarce, and antitumor activity of the extract obtained by the fermentation of the orange waste using the endophytic fungus *Diaporthe sp.* Following the fermentation and its extraction process, the fermented, the orange oil and the standards of (R)-(+)-limonene and (1S-2S-4R)-+-limonene-1,2-diol were tested to antitumoral activity with neuroblastoma cell line IMR-32 and antioxidant activity in macrophages (cell line RAW 267) analyzing superoxide anion (O_2) hydrogen peroxide (H_2O_2), phagocythosis and neutral red uptake. Overall, the fermented extract showed the best results in all of the tests when compared to the other treatments. In the antitumoral activity, the fermented extract showed an inhibition rate of 43.45% at the concentration of 2000 ppm and it was highly significant when compared either to the control or the orange extract ($p< 0.01$). The phagocytosis analysis of the macrophages resulted in a significant reduction ($p< 0.01$) of 38.53% in comparison to the control with the addition of the fermented extract (2000 ppm) and for the neutral red uptake the addition of the fermented extract showed a reduction of 36.30% ($p< 0.01$ in comparison to the control and $p< 0.05$ in comparison to the orange waste extract). The results of the superoxide anion analysis resulted in significant results for all the treatments ($p< 0.01$), while for the analysis of the hydrogen peroxide, the only treatment that present significant results was the fermented extract (2000 ppm), with a reduction of 26.40% ($p< 0.01$). The cytotoxicity assays performed with *Artemia salina* also showed positive results, showing that after the fermentation process the toxicity of the orange waste extract reduced from a LC50 of 432.54 μg/mL to 1676.86 mg/mL. These results suggest the application of the fermented extract in the control of the oxidative burst against inflammatory and auto-imune diseases and also showed the potential of the fermented extract against neuroblastoma cells. In this study we were successful to increase the bioactive properties already reported by the essential oil of orange.

Keywords: Orange essential oil, Antitumoral, Immunomodulatory, Macrophage, Solid state fermentation.

1. Introduction

The study of bioactivities of essential oils is a field in constant expansion today. Among the sources of essential oil, the orange peel stands out with approximately 95 % of limonene content (Danielski *et al.*, 2008; Bier *et al.,* 2016). Citrus fruits have high concentrations of limonene (Arce *et al..*, 2007, Diaz; Espinosa, Brignole, 2004; Yadava *et al*., 2004; Steinbrecher *et al.*, 1999). Several studies confirm the essential oil properties of orange and peel as antifungal activity (Velazquez - Nunez *et al*., 2013), anti-inflammatory effects (Gosslau *et al.* , 2014), antioxidant (Lu *et al.*, 2012; Chen *et al.*, 2012), antitumoral (Kaur; Kaur, 2015) and insecticide (Ezeonu *et al.*, 200; El- akhal *et al.*, 2015).

Limonene and its derivatives already have a number of reported bioactive properties: antioxidant activity and antigenotoxic (Bacanli *et al.*, 2015.), Effects on prevention and improvement of dyslipidemia and hyperglycemia in mice (Jing *et al.*, 2013), inhibition of angiogenesis, metastasis and cell death in human colon cancer cells by d-limonene, orange (Murthy *et al*., 2012), anxiolytic effects in mice (Lima *et al*., 2013), inhibitory activity against leishmania parasites *in vitro* and *in vivo* (Arruda *et al*., 2009), prevention and treatment of breast cancer (Miller *et al*., 2011) among others.

Some derivatives of the limonene already have their bioactive properties established. The carvone has antifungal and antibacterial properties (Carvalho; Fonseca, 2006). The perillyl alcohol (PA) is an isolated monoterpene essential oils of mint, cherry and celery seeds, among other plants. Studies in animals show that the PA is an effective chemotherapeutic agent in the regression of breast tumors, pancreas, liver and prostate, and chemopreventive agent for colon tumors, melanomas and neuroblastomas (Fischer *et al..*, 2005). The α-terpineol, one of the main derivatives of limonene, also recently presented studies indicating anticancer activities (Hassan *et al..*, 2010) and antioxidant activity Marostica Junior *et al*., 2009).

Macrophages are one of three types of phagocytes of the immune system, distributed widely to tissues, where they play a critical role in innate immunity. These cells are the mature form of monocytes circulating in the blood and continuously differentiate into macrophages after migrating to the tissues (Janeway et al.., 2002). Macrophages and other cells of the immune system produce mediators involved in the inflammatory

response such as cytokines, nitric oxide, and reactive oxygen species including superoxide anion (O_2-) and hydrogen peroxide (H_2O_2). These play a special role not only due to their antitumor activity, but also due to its ability to induce tissue damage. Because of the tissue damage that increased oxidative estress can cause, foods that exhibit antioxidant properties have been widely sought (Rubel *et al..,* 2010). The Immunomodulation is referred to as the regulation or control of the immunity which involves the enhancement or reduction in the immune responses (Tiwari *et al..*, 2014).

Another important aspect to be noted is the importance of the endophytic fungi with regards to biological properties and activities. Endophytic fungi have attracted much attention due to its high metabolic versatility (Deepika *et al.*, 2016). The potential of endophytes to demonstrate functional characteristics that justify the exploration of the use of pharmaceutical biotechnology is well established. However, these findings have not been translated into industrial processes for the commercial production of biopharmaceuticals (Kusari *et al.*, 2014).

According to Chapla (2010) the endophytic fungus *Diaporthe sp.* is an excellent producer of secondary metabolites, since 15 substances were identified with biological activity. Several other studies report bioactive properties of this fungus, as antimicrobial metabolites (Rakshitha *et al.,* 2013), antimicrobial and antitumor activity (Adelin *et al.*, 2011) and antimycotic activity (Rukachaisirikula *et al,*. 2008).

The main objective of this study was to evaluate the *in vitro* immunomodulation and antitumor activities of the extract obtained by the fermentation of the orange waste using endophytic fungus *Phomoposis sp*.

2 Material and Methods

2.1 Microorganism

A new strain of *Diaporthe* sp. was isolated from a reforestation area of the company Arauco Forest from Brazil, in Campo do Tenente (PR) was tested for limonene resistance and the ability to metabolize it (Bier *et al.*, 2011).

2.2 Solid State Fermentation

2.2.1 Pre-inoculum

The pre-inoculum preparation ocurred in Erlenmeyer flasks of 250 mL containing 50 mL of PDA. The Erlenmeyers were inoculated with a sterile platinum loop and incubated at 30 ° C for 168 hours. The flask was then subjected to magnetic stirring with saline solution during 15 minutes. The inoculum volume used was set to 3 mL, since this cultivation conditions *Diaporthe sp.* showed no spores for counting.

2.2.2 Inoculum

Diaporthe sp was cultivated for a period of 5 days in a natural orange residue extract based medium added ammonium sulfate (5g/L). The extract was prepared by the addition of 10 mL of water/g of dry substrate and placed in boiling water bath for 20 minutes. The extract was filtered and distributed in Erlenmeyer flasks of 125 mL containing 50 mL of medium. The medium was autoclaved for 15 minutes at 121°C. The suspension was inoculated into this medium and incubated 30°C at 120 rpm agitation. For the solid state fermentation, 5 mL of this inoculum was added to the solid medium.

2.2.3 Solid state fermentation

The orange residue containing orange bagasse and orange peel was be used as a substrate for fermentation in solid state due to its high content of limonene (5.11 g/ 100 g). In Erlenmenyers flasks of 250 mL, 20g of the dried orange residue were introduced. The initial water content was adjusted to 80% moisture. The initial pH of the medium was adjusted to 6.0. The particle size of the medium ranged from 0.8 to 2 mm. The culture media was sterilized by autoclaving at 121°C for 15 minutes. The fermentation was carried out for a period of 7 days at 30°C.

2.3 Fermented extract preparation

The extraction was performed in a portable extractor equipment for operations with liquefied gases developed by Oliveira (1997) and used for the extraction of terpenoids from the orange residue as described by Bier *et al.,* (2016). Liquefied petroleum gas (LPG), a mixture comprising butane and propane was used as the extractor solvent. The

composition of the gas used is propane (C_3H_8) 25% ± 5% and iso-butane + n-butane 75% ± 5% w/w. The solvent in the liquid phase is transferred into the equipment vessel where it comes into contact with 22 g of fermented medium. The extraction occurs using liquefied butane/propane.

Each of the two cycles of extraction used 15g of LPG (Volcano Isqueiros Ltda., São Paulo, SP, Brazil). The exposure time of the sample material was set to 20 minutes per cycle at 35ºC in an oven. The extract is transferred to a sealed vial using a needle attached to the extractor, without contact with the environment. Then, the LPG is evaporated at atmospheric pressure. All essays were performed in triplicates for each of the extraction methods.

2.4 Antitumoral activity

The test of the antiproliferative activity of tumor cells used the following media and solutions: Nutrient Mixture (DMEM) - $NaHCO_3$, antibiotic solution (Gibco®), fetal bovine serum (Gibco®); Trypsin (Sigma-Aldrich), MTT solution (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium hydrobromic) (Sigma-Aldrich) and PBS buffer. The methods for the assessment of anti-proliferative action of tumor cells were based from (Pick and Mizel, 1981) and used as adapted from Santos (2010).

The antitumor activity of the extracts was tested on neuroblastoma cell line IMR-32, kindly provided by St. Jude Children's Research (Memphis, USA). After thawing, the cells were transferred to a cell culture bottle with Nutrient Mixture culture medium (DMEM) with 10% fetal bovine serum and 1% antibiotic, followed by an incubation period of 24h at 37 °C. For the cell suspension, the cells were treated by adding 4 mL trypsin for 7 min, followed by the addition of 7 mL of culture medium, thus forming the cell suspension.

The standardization of cancer cells in the ELISA plate was checked by counting in a Neubauer chamber using 180 μL of the culture medium and 8.10^5 cancer cells in each well. A column was reserved for the blank containing only the culture medium and another column was reserved for the negative control (cancer cells without addition of compounds).

After incubating the ELISA plate for 24 hours 20 μL of the treatments at different concentrations of (R+-(+)-limonene and (1S,2S,4R)-(+)-limonene-1,2-diol (25, 100 and 500 ppm) and the extracts of orange waste and of fermented orange waste (50, 250, 1000 and 2000 ppm) were added in the culture medium. The treatments were removed and 150 μL of MTT (3.33g/ mL) was add in each well for 3 hours. After removal of the MTT, 150 μL of dimethyl sulfoxide was added. The absorbance was measured at 550 nm on microplate spectrometer. All operations occur under aseptic conditions. The results were expressed in absorbance (by $8x10^5$ cells/mL).

2.5 Antioxidant activity in macrophages

The antioxidant tests in macrophages were performed in order to verify the immunomodulatory effect of the standards and the extracts. The concentrations tested were based on the most significant results obtained by the MTT analysis

Macrophages (cell line RAW 267) were cultured in DMEM medium containing fetal bovine serum (10%) and antibiotic solution (1%). The cells were maintained at 37 °C in an CO_2 incubator at controllable conditions of humidity and carbon dioxide content (5%). The peripheral macrophages were evaluated for superoxide anion (O_2^-), hydrogen peroxide (H_2O_2), phagocytosis and lysosomal retention following the methodology based on Pick and Mizel (1981) and adopted by Rubel *et al.*., (2011).

To determine the production O_2^- and H_2O_2, phagocytosis and lysosomal volume ~~retention~~, macrophages aliquots (100 μL - cell density of $2x10^6$ cells/mL) were added to cell flat-bottomed 96 well plates and incubated for 24 hours. The limonene and limonene-1,2-diol (25, 100 and 500 ppm), the fermented extract and orange waste extract (50, 250, 1000 and 2000 ppm) were added and the incubation occurred for a period of 1h (superoxide anion and hydrogen peroxide) and 24 h (lysosomal retention and phagocytosis). After this period, the plates were washed with PBS buffer to remove non-adherent cells and 100 μL of PBS was added to the wells.

2.5.1 Superoxide Anion

The production of superoxide anion (O_2^-) was estimated by testing the reduction of nitro blue tetrazolium (NBT, Amresco) according to Bonatto *et al.,* (2004). Macrophages (100 μL) were incubated during 1 hour at 37 °C in the absence and presence of 10 μL of

phorbol myristate acetate (PMA, Sigma-Aldrich, final concentration of de 4 μM). Afterwards, 0.2% NBT was added and it was incubated for 30 minutes at 37 ºC in the dark. After the supernatant was discarded and the macrophages were fixed by the addition of 100 μL of methanol (50 %, Vetec) for 10 minutes. The supernatant was discarded and the plate was dried. Then 120 μL of KOH (2M) and 140 μL of dimethyl sulfoxide were added to the wells. After 30 minutes the absorbance of the sample was spectrophotometrically determined at 595 nm. The results were expressed in absorbance (by 10^6 cells/mL).

2.5.2 Hydrogen peroxide

The production of hydrogen peroxide (H_2O_2) was measured as described by Pick and Mizel (1981). This assay is based on the dependent conversion of the peroxidase of phenol red from horseradish in a colored compound mediated by the oxidation of phenol red by the H_2O_2. Macrophages (100 μL) were incubated in the absence and presence of 10 μL of phorbol myristate acetate in addition to the treatments for 1 hour. After the supernatant was discarded, they were incubated with glucose (5 mM), phenol red solution (0.56 mM) and peroxidase from horseradish (8.5 U/mL) in the dark for 30 minutes at 37 °C. The production of hydrogen peroxide was detected spectrophotometrically (620 nm) after the addition of 10 μL of NaOH 1M. The results were expressed in absorbance (per 10^6 cells/mL).

2.5.3 Phagocytosis

The phagocytosis was determined by adding 10 μL of Bio-Mos (Altech, $1x10^8$ particles/mL) colored with neutral red (Fluka) to each well containing 100 μL of macrophages. After the incubation (37 °C for 30 minutes) the macrophages were fixed with Baker formol-calcium (4% formaldehyde, 2% sodium chloride and 1% calcium acetate) for 30 minutes. After 30 minutes, the measurement occurred at 595 nm absorbance. The result was expressed in absorbance (by 10^6 cells/mL). The phagocythosis test was based on Bonatto *et al.,* (2004).

2.5.4 Uptake of neutral red

For the assessment of the lysosomal volume was measured the uptake of the cationic dye neutral red of the macrophages (Bonatto *et al.,* 2004). In each well 20 μL of 2%

neutral red was added and incubated for 30 minutes. After discarding the supernatant, 100 µl of extraction solution was added. After 30 minutes, the measurement occurred at 595 nm absorbance. The result was expressed in absorbance (by 10^6 cells/mL).

2.6 Toxicity essays

For the analysis of the toxicity of the compounds, the modified method of Solis *et al.*. (1993), already used by Gollo *et al.,* (2016), was performed. It was incubated 0.025 g of brine shrimp (*Artemia salina*) cysts in a becker. The sterile artificial sea water was prepared using Aquasalt (41.4 g/L) of Aqua ONE ™ and adjusted to pH 8.5 under constant aeration for 48 hours (in light). After hatching, the free *nauplii* were collected from the lighter portion of the incubation chamber and used for the test. Ten nauplii were collected via a micropipette (10 µL) and placed in each well (24- microwell plate) containing 1 mL of sterile artificial sea water. Limonene, and limonene-1,2-diol were evaluated for their toxicity with concentrations ranging from 62.5 µg/mL to 4000 µg/mL. The orange residue extract and the fermentation extract were tested in concentrations varying from 125 µg/mL to 4000 µg/mL. These concentrations were add to each of the wells containing 10 *nauplii* and the plates stayed in the dark for 24 h. The *nauplii* were then evaluated and the LD_{50} was calculated based on the mortality rate of each of the concentrations used.

The LC50 (lethal concentration, 50%) and 95% confidence intervals were calculated by Trimmed Spearman-Karber method by using TSK software version 1.5 (Usepa, 1990). The statical significance was verified by the Tukey's method for multiple comparison.

3. Results and Discussion

3.1 Antitumoral activity using cell viability assays by MTT

The MTT assays were performed for the standards (R)-(+)-limonene and (1S,2S,4R)-(+)-limonene-1,2-diol and for the extracts of orange (OE) waste (peel and pulp) and the extract of the fermented orange waste (FE).

The results of the MTT assays show a slight decrease of the viability of neuroblastoma cell line IMR-32 in the presence of the orange waste extract (OE) as seen in Figure 1. However, it is notable that the increase of concentration of the extract did not affect the cell proliferation. According to the Tukey's method for multiple comparisons, there was

no statistical difference in the cell viability for any concentration of orange waste extract used, but they were, indeed, different from the control (for control and OE 1000, $p<0.01$). The absorbance obtained with 1000 ppm was 0.5013 nm in comparison to 0.6678 nm of the control, which means an inhibition rate of 24.92% of the cell proliferation.

The fermented extract (2000 ppm) resulted in higher inhibitions than the ones obtained by the orange waste extract ($p< 0.01$). The Figure 1 shows that the increase in the concentration of the fermented extract leads to a higher inhibition (FE 1000 and FE 2000 $p<0.01$). With all the concentrations tested the fermented extract was significant in comparison to the control (for control and FE 50, $p<0.01$). The highest inhibition was obtained with the fermented extract at the concentration of 2000 ppm, with an inhibition rate of 43.45%.

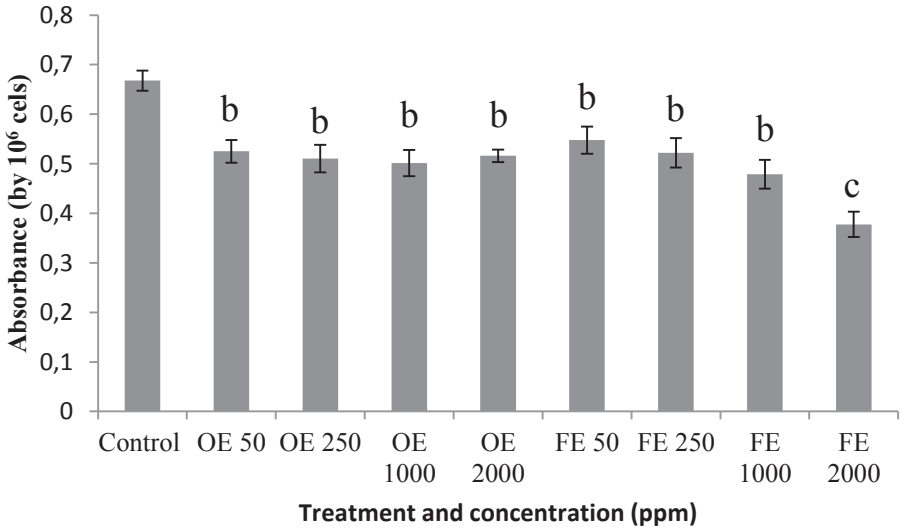


Figure 1: MTT analysis of the extract treatments. OE = Orange waste extract. FE = Fermented extract. For b and control $p<0.05$, for b and c $p<0.05$.

The cell viability of the neuroblastoma cell line IMR-32 was slightly reduced either in the presence of limonene, either in the presence of limonene-1,2-diol (Figure 2). Both inhibitions were significant when compared to the control (for control and L25, $p<0.01$; for control and Ld 25, $p<0.01$). The increase of limonene concentrations did not lead to a higher inhibition and the highest inhibition was obtained with limonene at the concentration of 25 ppm, with 0.5583 absorbance/10^6 cells/mL in comparison to 0.6666

absorbance/10^6 cells/mL of the control, with an inhibition rate of 16.25%. The lowest viability in the presence of limonene-1,2-diol was found after the concentration was increased for 500 ppm, with 0.5260 absorbance/10^6 cells/mL comparison to 0.6666 absorbance/10^6 cells/mL of the control, which means an inhibition rate of 21.09%.

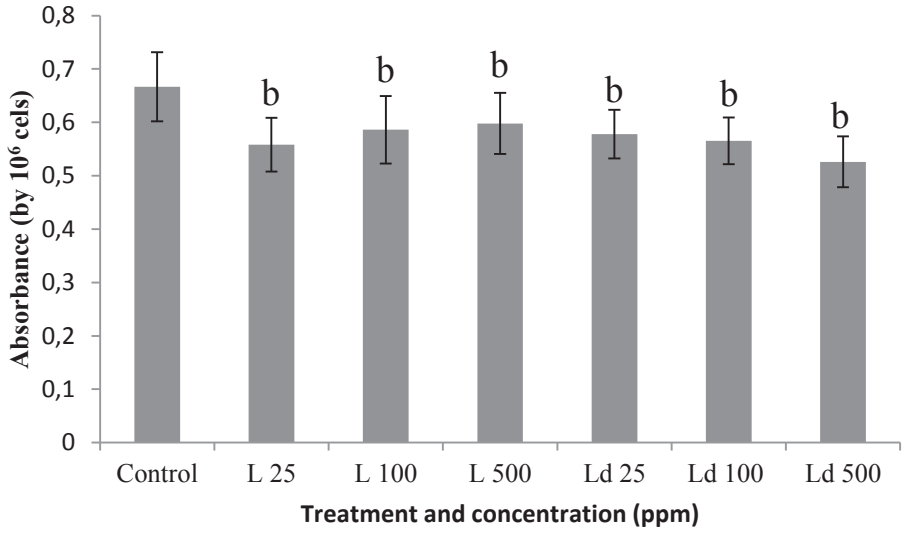


Figure 2: MTT analysis of the standard treatments. L = Limonene. Ld = Limonene-1,2-diol.

Both the fermented extract and the standard compound limonene-1,2-diol have showed higher antitumor activity than their precursors, however, the results obtained with the extracts can not be only fully justified by the presence of limonene and limonene-1,2-diol. The orange extract had higher tumor inhibition than the limonene itself, which means that other terpenes at low concentrations in the extract represent a great contribution for the activity. In the fermented extract it is even more evident. While the limonene-1,2-diol reached an inhibition rate of 21.09%. With 500 ppm of concentration, the fermented extract reached 21.81% of inhibition with only 250 ppm.

Limonene has been reported several times for its cytotoxicity against tumor cells (Murray, 2013; Poon *et al.,* 1996; Roberto *et al.,* 2009). The concentration values, however, are quite variable depending on the cell line tested. Guo *et al.*, (2006) tested the effects of D-limonene on leukemia cells and obtained a IC_{50} of 0.75 mmol, while Bacanli *et al..*, (2015) obtained a IC_{50} 1265 μM in Chinese hamster fibroblast (V79) cells. Concerning the use of extracts, Murthy *et al..*, (2012) obtained good results with concentrations up to 100 ppm of orange oil on human colon cancer cells, while Doll-

Boscardin *et al.*., (2012) tested concentrations up to 300 ppm of *Eucalyptus* extract against murine macrophage tumor, cervical cancer cells and T leukemia cells. It is notable that most authors used lower concentrations of extracts and standards and obtained the IC_{50} with lower concentrations of treatments. This difference can be attributed to the virulence and resistance of the neuroblastoma tumor cells. Among childhood malignancies, it has one of the worst survival rates (Acton, 2013).

It should be noted that the limonene and the orange waste extract were used as reference to compare the activity of the limonene-1,2-diol and the fermented extract. The limonene-1,2-diol has not yet been reported as a compound showing antitumor activity, but the fermentation extracts presented better results than the orange waste extract. The limonene-1,2-diol has been, however, related as a metabolite transformed from limonene in cases of human cancer (Crowell *et al.,* 1994; Poon *et al.,* 1996) what already relates it to antitumor activity. Orange extracts have been highly discussed recently for its antioxidant, antitumor and immunomodulatory capacity (Murthy *et al.,* 2012; Gosslau *et al*., 2014; Lu *et al.,* 2012; Chen *et al*., 2012; Kaur; Kaur , 2015).

It is clear that the limonene-1,2-diol contributes to the antitumor activity along with limonene, however, the wide variety of terpenes produced in the fermented extract in addition to the limonene-1,2-diol and the limonene that is still present, may play an important role in the biological activity here. Selvi *et al*., (2015) verified the cytotoxic activities of a terpenic essential oil in breast cancer cells and this essential oil included among them camphor, limonene, naphthalene, valencene, caryophyllene, α-pinene, camphene and terpinen-4-ol, some of them, among our fermentation products. Jaafari *et al*., (2012) concluded that carveol and isopulegol had significative effects on cell cycle of tumor cells, while Bicas *et al.* (2011) have showed results with α-terpineol, limonene and carvone against different tumor cell lines. The total activity of the fermented extract may be also related to the presence of other compounds produced by *Diaporthe sp.* already related to bioactivity properties in the literature. Orlandelli *et al.,* (2017) have connected the antiproliferative effect of *Diaporthe sp.* against cancer cells to the production of glucans, while Orlandelli *et al.,* (2016) have discussed the role of exopolysaccharides produced by the same group of fungi in antitumoral effects. Therefore, once terpenoids can be used due to their bioactivity and *Diaporthe sp.*

produces different categories of bioactivity compounds, in this study we have the effects of a fermented extract that focus on the properties of the aroma produced with the use of a natural bioactivity compound producer fungus.

3.2 Phagocytosis and Uptake of neutral red – cationic vesicles content

The phagocytosis analysis of the macrophages had not significant results for most of the treatments tested (figure 3). With the addition of limonene ($p>0.05$) there was a slightly reduction of the phagocytosis. The same occurred with the addition of limonene-1,2-diol ($p>0.05$). The addition of the orange waste extract did not result in significant results as well ($p>0.05$). However, in the presence of the fermentation extract the phagocytosis showed a significant reduction ($p<0.01$) with a reduction of 38.53%.

The effects on the phagocytosis may be little related to limonene or limonene-1,2-diol, instead, they may be attributed to the products from fermented orange waste and terpenoids verified on the GM-CS analysis on Chapter 4 and absent in the orange waste extract.

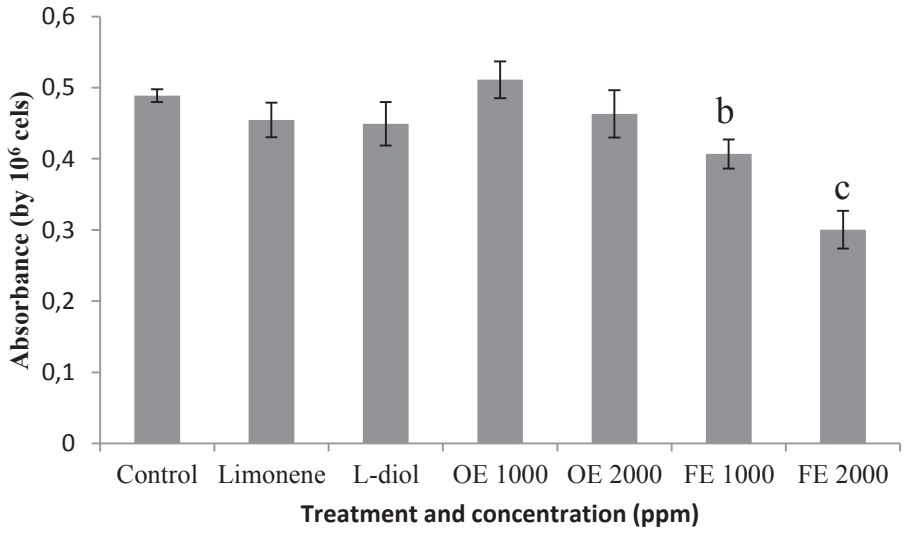


Figure 3: Phagocytosis analysis on macrophages. OE = Orange waste extract; FE = Fermentation extract; L-diol = Limonene-1,2-diol.

For the uptake of cationic dye neutral red, which concentrates in lysosomes, analysis neither the limonene or the limonene-1,2-diol showed results different from the control ($p>0.05$) as shown in Figure 4. The most significant results was obtained with the addition of the fermentation extract (2000 ppm), with a reduction of 36.30% in the

lysossomal volume ($p<0.01$). The orange waste extract in the concentration of 2000 ppm had significant results as well ($p<0.01$), with a reduction of 20.95% in the lysossosmal uptake neutral red. It is important to point that the reduction of the lysossomal volume by the fermentation extract was significant when compared to the orange waste extract as well ($p<0.05$).

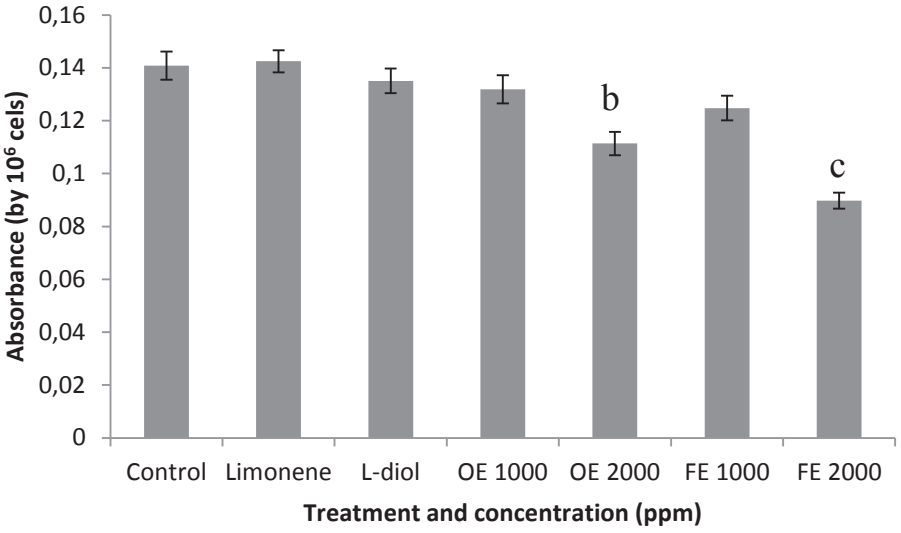


Figure 4: Neutral red uptake of the macrophages. OE = Orange waste extract; FE = Fermentation extract; L-diol = Limonene-1,2-diol.

Phagocytosis is widely available for assessing the immunomodulatory activity. It is the primary defense mechanism against any foreign bodies entering the body, which is offered by neutrophils and macrophages (Gupta et al*et al.*., 2010). Phagocytic cells become the center of inflammatory lesions partly because of release of substances previously sequestered within lysosomes (Zurier, 1976). The reduction of the phagocytosis and the neutral red uptake is very important in diseases where inflammatory and auto-imune processes take place.

3.3 Hydrogen Peroxide (H_2O_2) and superoxide anion radical (O_2-) production

The analysis of the superoxide anion production by the macrophages resulted in significant results obtained by different concentrations of each of the compounds and extracts (Figure 5). All the results were significant higher than values showed in the control ($p<0.01$). Although the highest values were showed by the orange waste extract (increase of 85.65% in comparison to the control), these values are close to the obtained

by limonene-1,2-diol (increase of 76.93%) and by the fermentation extract (increase of 85.42%), with no significant difference between the fermentation extract and the orange waste extract ($p > 0.05$). It is important to point, that the concentration of limonene-1,2-diol tested was 500 ppm, while the extracts were tested for 1000 ppm and 2000 ppm. The most notable result is the difference between the superoxide anion production in the presence of limonene and in the presence of limonene-1,2-diol ($p<0.01$). In the presence of limonene the production of superoxide anion was 1.4908 absorbance/10^6 cells/mL while in the presence of the limonene-1,2-diol it increase to 2.3910 absorbance/10^6 cells/mL.

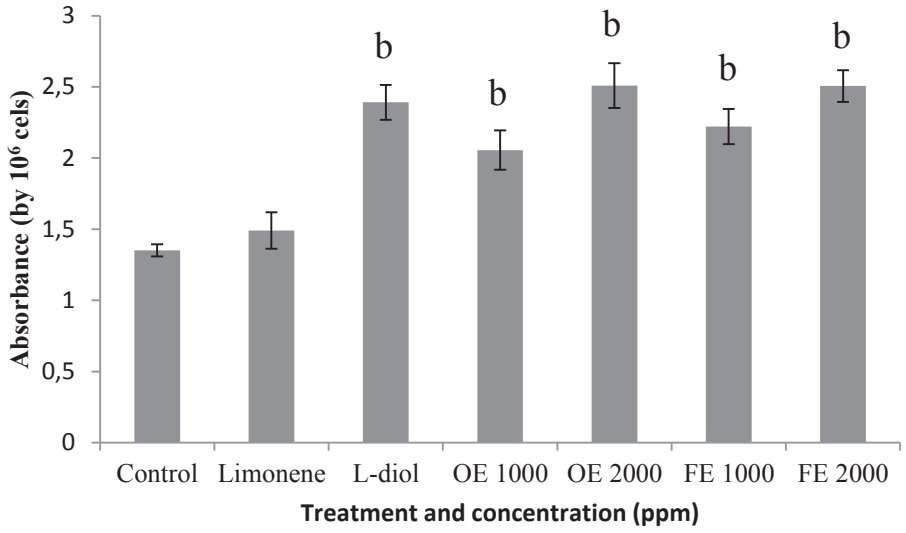


Figure 5: Superoxide anion production. OE = Orange waste extract; FE = Fermentation extract; L-diol = Limonene-1,2-diol.

The results suggests that both the presence of limonene and limonene-1,2-diol (in the fermentation extract) contribute to increase the production of superoxide anion, however, the limonene showed a minor contribution. The presence of limonene-1,2-diol in the fermented extract can explain the production of superoxide anion, although it is not present in the orange waste extract. Lastly, the activity showed by the orange waste extract may be explained by the presence of other monoterpenoids in its composition. The results regarding the limonene activity are in accordance with Maróstica Junior *et al.* (2009) who verified that limonene stimulated the formation of the O_2-. It was also verified, however, that carvone, perillyl alcohol and α-terpineol inhibited its formation. Our results regarding the extracts are very pertinent, once that many articles concluded

that their extracts had little effect on the O_2- or H_2O_2 production (Maróstica Junior *et al.*. 2009; Rubel *et al..,* 2010; Rubel *et al.,* 2011).

The analysis of the hydrogen peroxide produced by the macrophages revealed that the fermented extract at the concentration of 2000 ppm had significantly reduced the concentration of the hydrogen peroxide in comparison to the control and the other treatments ($p<0.01$) as seen in the Figure 6. The inhibition of the production of the hydrogen peroxide with the addition of the fermentation extract reached 26.40%, while the other treatments have not showed any significate difference in the results ($p>0.05$). These results are consistent with the results obtained with the phagocytosis analysis. The hydrogen peroxide production has seen to be little to not related to the presence of limonene and limonene-1,2-diol and its reduction in the extract of fermented orange waste could be explained by the activity of other fermentation products other than the limonene-1,2-dil and the limonene already present.

According to (Rubel *et al.*., 2010) hydrogen peroxide (H_2O_2) and reactive oxygen species, including superoxide anion (O_2) are important mediators involved in the inflammation response. Their excessive production can increase in the oxidative burst can induce severe tissue damage. Therefore, an extract that shows the ability to increase their production or limit them is very promising.

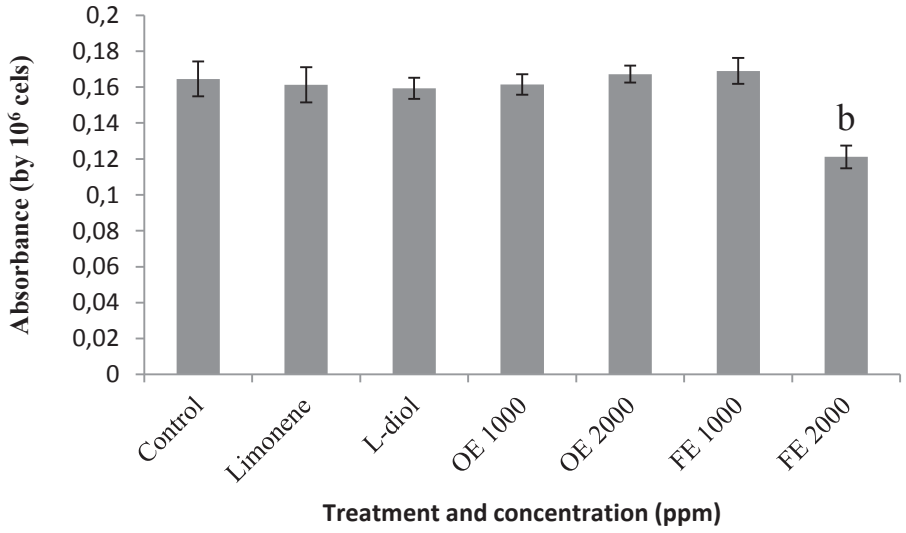


Figure 6: Hydrogen peroxide produced. OE = Orange waste extract. FE = Fermentation extract.

For the immunomodulatory effects in macrophages, the fermentation extract showed the highest activities in all of the experiments. While it reduced the phagocytosis, neutral red uptake and the hydrogen peroxide production, it caused an increase in the production of superoxide anion radical. The reduction of the neutral red uptake, phagocytosis and hydrogen peroxide production were already expected, since the extracts and standards already showed important results in the antioxidant capacity in vitro as seen in the Chapter 3.

3.4 Toxicity assays

The results of the toxicity test, showed an LC_{50} of 1676.86 mg/mL for the limonene-1,2-diol (Table 1). This value above 1000 µg/ mL is considered non-toxic (Nguta *et al.*, 2012) and less toxic than limonene (432.54 µg/mL), suggesting the possibility of using this compound as a flavor component. Hence the main compounds of the fermented extract are limonene-1,2-diol and limonene, and the first is the limonene biotransformation product, the lower toxicity of the limonene-1,2-diol contributes for the nontoxicity of the fermented extract.

The fermentation extract also had a significant decrease in its toxicity. While the orange waste extract had results similar to the one presented by the limonene, but more toxic (LC_{50} of 276.94 mg/mL), the fermented extract had a pretty low toxicity (LC_{50} of 3017.34 mg/mL),

Table 1: *Artemia salina* toxicity tests for limonene, limonene-1,2-diol, orange residue extract and the fermentation extract

Toxicant	LC50 (mg/L)	95% Lower Confidence	95% Upper Confidence
Limonene	432.54	330.69	565.77
Limonene-1,2-diol	1676.86	1259.22	2233.02
Orange residue extract	276.94	193.94	397.31
Fermentation extract	3017.34	2202.88	4132.93

Parra *et al.,* (2001) reported a good correlation between the in vivo and the in vitro tests with *Artermia salina* ($r = 0.85$ $p < 0.05$), regarding this method as a useful tool for predicting oral acute toxicity in extracts.

4. Conclusion

The variety of analysis performed regarding the antitumor activity against neuroblastoma cell line and the immunomodulatory effects in the antioxidant activity in macrophages suggests a great potential for the use this fermented extract in the medical field. The antitumor activity of the fermented extract showed an inhibition rate of 43.45% at the concentration of 2000 ppm and it was highly significant when compared to either the control or the orange extract ($p < 0.01$).

The phagocytosis analysis of the macrophages resulted in a significant reduction ($p< 0.01$) of 38.53% in comparison to the control with the addition of the fermented extract (2000 ppm), while the other treatments resulted in not significant results. For the neutral red uptake, the most significant results ($p< 0.01$ in comparison to the control) were obtained with the addition of the fermentation extract at 2000 ppm (36.30%). In this test, the orange waste extract showed a significate reduction (when compared to the control as well ($p< 0.01$) with a reduction of 20.95% in the neutral red uptake, but less significate than the obtained with the fermented extract ($p< 0.05$). These results of the fermented extract suggest that this extract may take place against inflammatory and autoimmune diseases.

The results of the superoxide anion analysis resulted in significant results for all the treatments ($p< 0.01$), with the exception of limonene, while for the analysis of the hydrogen peroxide, the only treatment that present significant results was the fermented extract (2000 ppm), with a reduction of 26.40% ($p< 0.01$). These results once again affirm the potential of the fermented extract to control the oxidative burst in the inflammatory response generated by these compounds.

At last, the cytotoxicity assays performed with *Artemia salina* corroborate positively with the results, showing that the fermented extract has more bioactive potential than the orange waste extract and has less toxicity (LC50: 1676.86 mg/mL) than its precursor (LC50: 432.54 µg/mL).

References

Acton, Q. A. Neuroblastomas: New Insights for the Healthcare Professional. Atlanta: Scholarly Editions, 2013

Adelin, E. Servy, C. Cortial, S. Lévaique, H. Martin, M.T. Retailleau, P. Le Goff, G. Bussaban, B. Lumyong, S. Ouazzani, J. Isolation, structure elucidation and biological activity of metabolites from Sch-642305-producing endophytic fungus *Phomopsis* sp. CMU-LMA, Phytochemistry 72 (18) (2011) 2406-2412

Arce, A. Pobudkowskal, A. Rodríguez, O. Soto, O. Citrus essential oil terpenless by extraction using 1-ethyl-3-methylimidazolium ethylsulfate ionic liquid: Effect of the temperature, Chemical Engineering Journal 133 (2007) 213-218.

Arruda, D. C., Miguel, D. C., Yokoama-Yasunaka, J. K. U., Katzin, A. M., Uliana, S. R. B. Inhibitory activity of limonene against Leishmania, Biomedicine & Pharmacotherapy 63 (2009) 643-649

Bacanli, M., Basaran, A. A., Basaran, N. The antioxidant and antigenotoxic properties of citrus phenolics limonene and naringin, Food and Chemical Technology 81 (2015) 160-170

Bicas, J. L. Neri-Numa, I. A. Ruiz, A. L. T. G. De Carvalho, J. E. Pastore, G. M. Evaluation of the antioxidant and antiproliferative potential of bioflavors, Food and Chemical Toxicology 49 (2011) 1610–1615

Bier, M. C. J., Medeiros, A. B. P., De Oliveira, J. S., Côcco, L. C., Costa, J. L., Soccol, C. R. Liquefied gas extraction: A new method for the recovery of terpenoids from agroindustrial and forest wastes. The Journal of Supercritical Fluids 110 (2016) 97-102.

Bier, M. C. J., Poletto, S., Soccol, V. T., Soccol, C. R. Medeiros, A. B. P. Isolation and Screening of Microorganisms with Potential for Biotransformation of Terpenic Substrates, Brazilian Archives of Biology and Technology 54 (2011) 1019-1026

Bonatto, S. J. R.; Folador, A. Aikawa, J.; Yamazaki, R. K.; Pizatto, N.; Oliveira, H. H.; Vecchi, R.; Curi, R.; Calder, P. C.; Fernandes, L. C. Lifelong exposure to dietary fish oil alters macrophage responses in Walker 256 tumor-bearing rats, Cell Imunology 231, n. (1-2). (2004) 56-62.

Carvalho, C. C. R. de. Fonseca, M. M. R. da. Biotransformation of terpenes, Biotechnology Advances 24 (2) (2006) 134–142

Chen, Z. Chu, H. Chyau, C. Chu, C. Duh, P. Protective effects of sweet orange (Citrus sinensis) peel and their bioactive compounds on oxidative stress, Food Chemistry 135 (2012) 2119–2127

Crowell, P. L. Elson. C. E. Bailey. H. H. Elegbede, A. Haag, J. D. Gould, M. N. Human metabolism of the experimental cancer therapeutic agent d-limonene, Cancer Chemotherapy and Pharmacology 35 (1994) 31-37

Danielski, L. Brunner, G. Schwänke, C. Zetzl, H. Hense, C. Donoso, J. P. M. Deterpenation of mandarin (Citrus reticulata) peel oils by means of countercurrent multistage extraction and adsorption/desorption with supercritical CO2, J. Supercritical Fluids 44 (2008) 215-324

Deepika, V.B. Murali, T.S. Satyamoorthy, K. Modulation of genetic clusters for synthesis of bioactive molecules in fungal endophytes: A review, Microbiological Research 182 (2016) 125–140

Diaz, S. Espinosa, S. Brignole, E. A. Citrus peel oil deterpenation with supercritical fluids optimal process and solvent cycle design, J. Supercritical Fluids 35 (2005) 49-61

Döll-Boscardin, P. M. Sartoratto, A. Maia, B. H. L. N. S. Paula, J. P. de, Nakashima, T. Farago, P. V. Kanunfre, C. C. In Vitro Cytotoxic Potential of Essential Oils of Eucalyptus benthamii and Its Related Terpenes on Tumor Cell Lines, Evidence-Based Complementary and Alternative Medicine 2012 (2012) 1-8.

El-Akhal, F. Lalami, A. E. O. Guemmouh, R. Larvicidal activity of essential oils of Citrus sinensis and Citrus aurantium (Rutaceae) cultivated in Morocco against the malaria vector Anopheles labranchiae (Diptera: Culicidae), Asian Pacific Journal of Tropical Disease 5 (2015) 458-462

Ezeonu, F. C. Chidume, G. I. Udedi, S. C. Insecticidal properties of volatile extracts of orange peels, Bioresource Technology 76 (2001) 273-274

Fischer, J. S. G. Mota e Silva, M. S. Paschoal, M. Carvalho, P. C. Carvalho, M. G. C. Efeito do álcool perílico na expressão gênica de células de adenocarcinoma de pulmão humano, Jornal Brasileiro de Pneumologia 31 (2005) 511-515

Gollo, A. L. Silva, A. L. L. de, Lima, K. K. D. de. Costa, J. L. Camara, M. C. Biasi, L. A. Rodrigues, C. Vandenberghe, L. P. S. Soccol, V. T. Socco, C. R. Developing a Plant Culture Medium Composed of Vinasse Originating from *Haematoccus pluvialis* Culture, Pakistan Journal of Botany 48 (1) (2016) 295-303.

Gosslau A. Chen, K. Y. Ho, C. Li, S. Anti-inflammatory effects of characterized orange peel extracts enriched with bioactive polymethoxyflavones, Food Science and Human Wellness 3 (2014) 26-35

Guo, X. M. Lu, Q. Liu Z. J. Wang, L. F. Feng, B. A. Effects of D-limonene on leukemia cells HL-60 and K562 in vitro, Journal of Experimental Hematology 14 (4) (2006) 692-695

Gupta, S. Pramanik, S. Tiwari, O. Thacker, N. Pande, M. Upmanyu, N. Immunomodulatory activity of gymnema sylvestre leaves, The Internet Journal of Pharmacology 8 (2) (2010) 1p.

Hassan, S. B. Gali-Muhtasib, H. Goransson, H. Larsson, R. Alpha Terpineol: A Potential Anticancer Agent which Acts through Suppressing NF-κB Signalling, Anticancer Researches 30 (2010) 1911-1919

Jaafari, A. Tilaoui, M. Mouse, H. A. M'bark, L. A. Aboufatima, R. Chait, A. Lepoivre, M. ZYad, A. Comparative study of the antitumor effect of natural monoterpenes: relationship to cell cycle analysis, Brazilian Journal of Pharmacognosy 22(3) (2012) 534-540

Janeway, C.A. Travers, P. Walport T. M. Capra, J.D. Imunobiologia. O sistema imunológico na saúde e na doença. 5ª ed. Porto Alegre: ArtMed, 2002, 767 p

Jing, L. Zhang, Y. Fan, S. Gu, M. Guan, Y. Lu, X. Huang, C. Zhou, Z. Preventive and ameliorating effects of citrus D-limonene on dyslipidemia and hyperglycemia in mice with high-fat diet-induced obesity, European Journal of Pharmacology 715 (2013) 46–55

Kaur, J. Kaur, G. An insight into the role of citrus bioactives in modulation of colon cancer, Journal of Functional Foods 13 (2015) 239-261

Kusari, S. Singh, S. Jayabaskaran, C. Biotechnological potential of plant-associated endophytic fungi: hope versus hype, Trends in Biotechnology 32 (6) (2014) 297-303

Lima, N. G. P. B., Sousa, D. P. de, Pimenta, F. C. F., Alvez, M. F. Souza, F. S. de, Macedo, R. O., Cardoso, R. B., Morais, L. C. S. L. de, Diniz, M. F. F. M., Almeida, R. N. de. Anxiolytic-like activity and GC–MS analysis of (R)-(+)-limonene fragrance, a natural compound found in foods and plants, Pharmacology, Biochemistry and Behavior 103 (2013) 450-454

Logarto, P. A. Silva, Y. R. Guerra, S. I. Iglesias, B. L. Comparative study of the assay of Artemia salina L. and the estimate of the medium lethal dose (LD50 value) in mice, to determine oral acute toxicity of plant extracts, Phytomedicine 8 (5) (2001) 395-400.

Lu, Y. Zhao, S. Zhang, J. Zhang, H. Xie, Z. Cai, G. Jiang, W. Effect of orange peel essential oil on oxidative stress in AOM animals, International Journal of Biological Macromolecules 50 (2012) 1144-1150

Maróstica Junior, M. R. Rocha e Silva, T. A. A. Franchi, G. C. Nowill, A. Pastore, G. M. Hyslop, S. Antioxidant potential of aroma compounds obtained by limonene biotransformation of orange essential oil, Food Chemistry 116 (2009) 8–12

Maróstica Júnior, M. R., Silva, T. A. A. R., Franchi, G. C., Nowill, A., Pastore, G. M., Hyslop, S. Antioxidant potential of aroma compounds obtained by limonene biotransformation of orange essential oil, Food Chemistry 116 (2009) 8-12

Miller, J. A. Thompson, P. A. Hakim, I. A. Chow, H. S. Thomson, C. A. D-Limonene: a bioactive food component from citrus and evidence for a potential role in breast cancer prevention and treatment, Oncology Review 5 (2011) 31-42

Murray, M. T. The Complete Book of Juicing, Revised and Updated: Your Delicious Guide to Youthful Vitality. Clarkson Potter (2013) 384 p

Murthy, K. N. C., Jayaprakasha, G. K., Patil, B. S. D-limonene rich volatile oil from blood oranges inhibits angiogenesis, metastasis and cell death in human colon cancer cells, Life Sciences 91 (2012) 429-439

Nguta, J. M. Mbaria, J. M. Gakuya, D. W. Gathumbi, P. K. Kabasa, J. D. Kiama, S. G. Evaluation of Acute Toxicity of Crude Plant Extracts from Kenyan Biodiversity, using Brine Shrimp, Artemia salina L. (Artemiidae), The Open Conference Proceedings Journal 3 (2012) 30-34

Oliveira, J. S Extrator para Operação com Gases Liquefeitos, Brazilian Patent PI 9804199-1, May 23, 2000

Orlandelli, R. C. Vasconcelos, A. F. B. Azevedo, J. L. Silva, M. L. C. da. Pamphile, J. A. Screening of endophytic sources of exopolysaccharides: Preliminary characterization of crude exopolysaccharide produced by submerged culture of *Diaporthe sp*. JF766998 under different cultivation time, Biochimie Open 2 (2016) 33-40

Orlandelli, R. C. Silva, M. L. C. da. Vasconcelos, A. F. D. Almdeira, I. V. Vicentini, V. E. P. Prieto, A. Hernandez, M. D. D. Azevedo, J. L. Pamphilea, J. A. -(1→3,1→6)-d-glucans produced by *Diaporthe sp*. endophytes: Purification, chemical characterization and antiproliferative activity against MCF-7 and HepG2-C3A cells, International Journal of Biological Macromolecules 94 (2017) 431-437

Pick E., Mizel D. Rapid microassays for the measurement of superoxide and hydrogen peroxide production by macrophages in culture using an automatic enzyme immunoassay reader, Journal of Immunology Methods 46 (1981) 211-226

Poon, G. K. Vigushin, D. Griggs, L. J. Rowlands, M. G. Coombes, R. C. Jarman, M. Identification and characterization of limonene metabolites in patients with advanced cancer by liquid chromatography/mass spectrometry, Drug Metabolism & Disposition 24 (1996) 565-571

Rakshith, D. Santosh, P. Satisha, S. Isolation and characterization of antimicrobial metabolite producing endophytic *Phomopsis* sp. from Ficus pumila Linn. (Moraceae), International Journal of Chemical and Analytical Science 4 (3) (2013) 156–160

Roberto, D. Micucci, P. Sebastian, T. Graciela, F. Anesini, C. Antioxidant Activity of Limonene on Normal Murine Lymphocytes: Relation to H2O2 Modulation and Cell Proliferation, Basic & Clinical Pharmacology & Toxicology 106 (2009) 38-44

Rubel, R. Santa, H. D. S. Fernandes, L. C. Bonatto, S. J. R. Bello, S. Figueiredo, B. C. Lima Filho, J. H. Santos, C. A. M. Soccol, C. R. Hypolipidemic and antioxidant properties of Ganoderma lucidum (Leyss:Fr) Karst used as a dietary supplement, World Journal of Microbiology and Biotechnology 27 (2011) 1083-1089

Rubel, R. Santa, H. S. D. Bonatto, S. J. R. Bello, S. Fernandes, L. C. Bernardi, R. D. Gern, J. Santos, C. A. Soccol, C. R. Medicinal Mushroom Ganoderma lucidum (Leyss: Fr) Karst. Triggers Immunomodulatory Effects and Reduces Nitric Oxide Synthesis in Mice, Journal of Medicinal Food 13 (2010) 142-148

Rukachaisirikul, V. Sommart, U. Phongpaichit, S. Sakayaroj, J. Kirtikara, K. Metabolites from the endophytic fungus *Phomopsis* sp. PSU-D15, Phytochemistry 69 (3) 783-787

Santos, L. F. dos. Produção e Aplicação da levana de *Bacillus subtilis* natto em filmes isolados para liberação em cólon – específica de fármacos. 255 f. Dissertação (Mestrado em Biotecnologia) – Área agroindustrial, Universidade Estadual de Londrina, Londrina, 2010.

Selvi, M. T. Thirugnanasampandan, R. Sundarammal, S. Antioxidant and cytotoxic activities of essential oil of Ocimum canum Sims. from India, Journal of Saudi Chemical Society 19 (2015) 97-100.

Solis, P. N. Wright, C. W. Anderson, M.M. Gupta, M. P. Phillipson, J. D. A microwell cytotoxicity assay using *Artemia salina* (brine shrimp), Planta Médica. 59 (3) (1993) 250-252.

Steinbrecher, R. Hauff, K. Riissler, J. Diirr, M. Seufert, G. Monoterpene Emission from Valencian Citrus Belt, Spain Soils in Orange Plantations of the Spain. Physics and Chemistry of the Earth B. 24 (1999) 695-698

Tiwari, P. Mishra, N. Sangwan, N. Phytochemical and Pharmacological Properties of *Gymnema sylvestre*: An Important Medicinal Plant, BioMed Research International 2014 (2014) 1-18

Usepa, 1990. Trimmed spearman-karber (TSK) program version 1.5 ecological monitoring research division. Environmental Monitoring Systems Laboratory, USEPA, Cincinnati, OH.

Velázquez-Nuñez, M. J. Avila-Sosa, R. Palou, E. López-Malo, A. Antifungal activity of orange (Citrus sinensis var. Valencia) peel essential oil applied by direct addition or vapor contact, Food Control 31 (2013) 1-4

Yadava, A. R. Chauhan, A. S. Rekha, . M. N. Ramteke, R. S. Flavour quality of dehydrated lime [Citrus aurantifolia (Christm.) Swingle], Food Chemistry 85 (2004) 59-62

Zurier, R. B. Reduction of Phagocytosis and Lysosomal Enzyme Release From Human Leukocytes by Serum from Patients With Systemic Lupus Erythematosus, Arthritis and Rheumatism 19 (1) (1976) 73-78

CONSIDERAÇÕES FINAIS

O trabalho desenvolveu-se a partir de conhecimentos anteriores da biotransformação do limoneno pelo fungo isolado *Phomopsis sp.* (*Diaporthe)* e atingiu os objetivos inicialmente propostos. Importantes resultados foram obtidos em relação àrecuperação do extrato fermentado, na fermentação submersa utilizando meio sintético e natural, na fermentação no estado sólido, nos testes de atividade antioxidante e nos testes de atividade antitumoral e imunomoduladora.

Com foco nos produtos de biotransformação, verificou-se que o método de extração com gás liquefeito de petróleo utilizando um extrato portátil apresentou resultadores superiores e satisfatórios para o resíduo de laranja. Considerando que este resíduo era rico em terpenos e terpenóides, a extração foi considerada representativa emrelação ao objetivo pretendido. Este método foi utilizado nas extrações posteriores do material fermentado. Além da quantidade obtida, o método proposto apresentava-se como menos agressivo aos compostos obtidos, e portanto, mais estável, além disso, operfil qualitativo do extrato era mais completo com relação aos demais métodos.

A fermentação submersa antecedeu os estudos de fermentação no estado sólido, e foi realizada sob várias condições. Neste estudo, o perfil metabólico da cepa foi verificado através de mudanças no preparo do inóculo, de maneira que uma das metodologias promovia uma mudança da forma anamorfa (*Phomopsis)* da cepa para a telemorfa. A cepa apresentou diferenças metabólicas interessantes, com perfis qualitativos semelhantes, mas com alterações na concentração do composto majoritário, com ênfase na carvona, limoneno-1,2-diol e α-terpineol. Além do fato de não haverem até então resultados significativos com fungos endofíticos na biotransformação de limoneno, a mudança de um meio sintético para um meio natural baseado em extrato de resíduo de laranja obteve bons resultados na produção de limoneno-1,2-diol, além de apresentar outros derivados do limoneno como produtos minoritários da fermentação.

A fermentação no estado sólido é um processo que ainda não possuía registros na biotransformação do limoneno em seus derivados, apesar do resíduo de laranja já ter sido utilizado para outros objetivos. Por possuir uma grande quantidade de limoneno,

este meio constituiu a fonte ideal para este processo, utilizando-se o mesmo fungo isolado, *Phomopsis sp.* que demonstrou resultados interessantes na fermentação submersa. Na fermentação no estado sólido as concentrações obtidas de limoneno-1,2-diol e α-terpineol foram significativas. O extrato foi obtido através da extração com gás GLP e então testado para diversos métodos de atividade antioxidante, tais como CUPRAC, ORAC e DPPH. Os resultados obtidos com relação ao extrato do resíduo de laranja fermentado foram bastante significativos, principalmente em relação ao extrato do resíduo da laranja, que já é conhecido na literatura por seu potencial antioxidante. O extrato fermentado apresentou atividade antioxidante superior ao do resíduo de laranja nos métodos ORAC, CUPRAC e DPPH, assim como foi superior na concentração de fenóis totais. A atividade antioxidante do extrato fermentado foi, portanto, bastante significativa, sendo comparável a fontes de atividade antioxidante com potencial bastante superior ao do resíduo de laranja, como a casca de jabuticaba, no caso do ORAC.

Por fim, o extrato fermentado obtido também foi testado com relação a sua atividade antitumoral e imunomoduladora. A atividade antitumoral do extrato fermentado não só foi maior que o do extrato de resíduo de laranja, como apresentou resultados promissores para todos os testes de atividade antioxidante em macrófagos, levando a conclusão do potencial deste extrato no combate a reações inflamatórios e doenças auto-imunes. Estes testes revelaram que o extrato apresentava a capacidade de reduzir a retenção lisossomal, fagocitose e produção de peróxido de hidrogênio, ao mesmo tempo que sua presença resultou no aumento da atividade do ânion superóxido, um importante mediador da resposta imune. Além dos resultados obtidos com o extrato fermentado, os resultados obtidos com o limoneno-1,2-diol foram bastante expressivos, em alguns casos obtendo valores mais significativos em relação ao controle que o obtido pelo limoneno. A importância do conhecimento da atividade destes compostos se dá pela sua concentração no extrato fermentado, assim como a ausência de estudos de suas atividades na literatura.

REFERÊNCIAS

Abbas, A. K.; Lichtman, A. H. **Imunologia Celular e Molecular**, 5º ed. Rio de Janeiro: Elsevier, 2005.

Abraham, B. G.; Berger, R. G. Higher fungi for generating aroma compounds though novel biotechnologies. **Journal of Agricultural and Food Chemistry**, v. 42, p. 2344-2348, 1994.

Abrahão, M. R. E.; Molina, G.; Pastore, G. M. Endophytes: Recent developments in biotechnology and the potential for flavor production. **Food Research International**, v. 52, n.1, p. 367–372, 2013.

Abreu, L. M.; Costa, S. S.; Pfenning, L.; Takahashi, J. A.; Larsen, T. O.; Andersen, B. Chemical and molecular characterization of *Phomopsis* and *Cytospora*-like endophytes from different host plants in Brazil. **Fungal Biology,** v. 116, p. 249-260, 2012.

Acton, Q. A. **Neuroblastomas: New Insights for the Healthcare Professional.** Atlanta: Scholarly Editions, 2013.

Adams, A.; Demyttenaere J. C. R.; De Kimpe N. Biotransformation of (R)-(+)- and (S)-(-)-limonene to α-terpineol by *Penicillium digitatum* — investigation of the culture conditions. **Food Chemistry**, v. 80, p. 525-534, 2003.

Adams, RP, 2010. **Identification of essential oil components by gas chromatography/mass Spectrometry**, 4th ed. Carol Stream: Allured Publishing Corporation, 2010.

Adelin, E.; Servy, C.; Cortial, S.; Lévaique, H.; Martin, M. T.; Retailleau, P.; Le Goff, G.; Bussaban, B.; Lumyong, S.; Ouazzani, J. Isolation, structure elucidation and biological activity of metabolites from Sch-642305-producing endophytic fungus *Phomopsis* sp. CMU-LMA. **Phytochemistry**, v. 72, n.18, p. 2406-2412, 2011.

Ahamed, A.; Ahring, B.K. Production of hydrocarbon compounds by endophytic fungi Gliocladium species grown on cellulose. **Bioresource Technology**, v. 102, n. 20, p. 9718-9722, 2011.

Akacha, N. B.; Gargouri, M. Microbial and enzymatic technologies used for the production of natural aroma compounds: Synthesis, recovery modeling, and bioprocesses. **Food and Bioproducts Processing**, v. 94, p. 675–706, 2015.

Ali, R.; Mirza, Z.; Ashraf, G.; M. Kamal, M. A.; Ansari, S. A. Damanhouri, G. A. Abuzenadah, A. M. Chaudhary, A. G. Sheikh, I. A. New anticancer agents: recent developments in tumor therapy. **Anticancer Research**, v. 32, n. 7, p. 2999-3005, 2012.

Apak, R.; Güçlü, K.; Demirata, B.; Özyürek, M.; Çelik, S. E.; Bektaşoğlu, B.; Berker, I.; Özyurt, D. Comparative evaluation of various total antioxidant capacity assays applied to phenolic compounds with the CUPRAC assay. **Molecules,** v. 12, p. 1496-1547, 2007.

Arce, A.; Pobudkowskal, A.; Rodríguez, O.; Soto, O. Citrus essential oil terpenless by extraction using 1-ethyl-3-methylimidazolium ethylsulfate ionic liquid: Effect of the temperature. **Chemical Engineering Journal**, v.133, p.213-218, 2007.

Arruda, D. C.; Miguel, D. C.; Yokoama-Yasunaka, J. K. U.; Katzin, A. M., Uliana, S. R. B. Inhibitory activity of limonene against Leishmania. **Biomedicine & Pharmacotherapy**, v. 63, p. 643-649, 2009.

Ashish, S. Global flavors (food and beverages) market - size, share, trends, analysis, opportunities, growth, and forecast, 2014 – 2020. Portland: Allied Market Research,. 2015.

Assefa, A. D.; Ko, E. Y.; Moon, S. H.; Keum, Y-S. Antioxidant and antiplatelet activities of flavonoid-rich fractions of three citrus fruits from Korea. **3 Biotech**, v. 6, p. 1-10, 2016.

Bacanli, M.; Basaran, A. A.; Basaran, N. The antioxidant and antigenotoxic properties of citrus phenolics limonene and naringin. **Food and Chemical Technology**, v. 81, p. 160-170, 2015.

Badee, A. Z. M.; Helmy, S. A.; Morsy, N. F. S. Utilisation of orange peel in the production of α-terpineol by Penicillium digitatum (NRRL 1202). **Food Chemistry**, v. 126, p. 849–854, 2011.

Bain & Company. **Potencial de Diversificação da Indústria Química Brasileira** – Relatório 4 – aromas, sabores e fragrâncias. Rio de Janeiro: Bain & Company, 2014.

Barbosa, L. C. A. **Espectroscopia no Infravermelho na caracerização de Compostos Orgânicos**. Viçosa: Editora UFV, 2007.

Barnola, L. F.; Cedeño, A.; Hasegawa, M.; Intra-individual variations of volatile terpene contents in Pinus caribaea needles and its possible relationship to Atta laevigata herbivory. **Biochemical Systematics and Ecology**, v. 25, p. 707-716, 1997.

Bauer, K.; Garbe, D.; Suburg, H. **Common Fragrances and Flavor Materials**. Weinheim: Wiley-VCH, 2001.

Berger, R. G. Biotechnology as a source of natural volatile flavours. **Current Opinion in Food Science**, v. 1, p. 38–43, 2015.

Berger, R. G.; De Bont, J. A. M.; Eggink, G. M.; Da Fonseca, M. Gehrke, Gros, J-B. Keulen, F. V. Kings, U. Biotransformations in the Flavour Industry. In: SWIFT, K. A. D. **Current Topics in Flavours and Fragrances**. New York: Springer Netherlands, 1999, p.139-170.

Berger, R. G. **Flavours and Fragrances: Chemistry, Bioprocessing and Sustainability**. New York: Springer Berlin Heidelberg, 2007.

Berger, R. G., Biotechnology of flavours—the next generation. **Biotechnology** Letters, v. 11, p. 1651-1659, 2009.

Beta, T.; Duodu, K. G. Bioactive: antioxidants. In: WRIGLEY, C.; CORKE, H.; SEETHARAMN, K.; FAUBION, J. **Encyclopedia of Food Grains.** Oxford: Academic Pres, 2016, p. 277-282.

Bicas, J. K.; Quadros, C. P.; de. Néri-Numa, I. A.; Pastore, G. M. Integrated process for co-production of alkaline lipase and R-(+)-α-terpineol by Fusarium oxysporum. **Food Chemistry**, v. 120, n. 2, p 452–456, 2010.

Bicas, J. L.; Neri-Numa, I. A.; Ruiz, A. L. T. G.; De Carvalho, J. E.; Pastore, G. M. Evaluation of the antioxidant and antiproliferative potential of bioflavors. **Food and Chemical Toxicology**, v. 49, p. 1610–1615, 2011.

Bicas, J. L.; Fontanille, P.; Pastore, G. M.; Larroche, C. Characterization of monoterpene biotransformation in two pseudomonads. **Journal of Applied Microbiology**, v. 105, n. 6, p. 1991-2001, 2008.

Bicas, J. L.; Pastore, G. M. Isolation and screening of D-limonene-resistant microorganisms. **Brazilian Journal of Microbiology**, v. 38, p. 563-567, 2007.

Bier, M. C. J.; Medeiros, A. B. P.; De Oliveira, J. S.; Côcco, L. C.; Costa, J. L., Soccol, C. R. Liquefied gas extraction: A new method for the recovery of terpenoids from agroindustrial and forest wastes. **The Journal of Supercritical Fluids**, v. 110, p. 97-102, 2016.

Bier, M. C. J.; Poletto, S.; Soccol, V. T.; Soccol, C. R.; Medeiros, A. B. P. Isolation and Screening of Microorganisms with Potential for Biotransformation of Terpenic Substrates. **Brazilian Archives of Biology and Technology**, v. 54, p. 1019-1026, 2011.

Bier, M .C. J.; Medeiros, A. B. P.; De Oliveira, J. S.; Côcco, L. C.; Costa, J. L.; Soccol, C. R. Liquefied gas extraction: A new method for the recovery of terpenoids from agroindustrial and forest wastes. **The Journal of Supercritical Fluids**, v. 110, p. 97-102, 2016.

Bier, M. C. J.; Medeiros, A. B. P.; Soccol, C. R. Biotransformation of limonene by an endophytic fungus using synthetic and orange residue-based media. **Fungal Biology,** v.121, p. 137-144, 2017.

Block, G.; Patterson, B.; Subar, A. Fruit, vegetables, and cancer prevention: a review of the epidemiological evidence. **Nutrional Cancer**, v. 18, p. 1–29, 1992

Bonatto, S. J. R.; Folador, A. Aikawa, J.; Yamazaki, R. K.; Pizatto, N.; Oliveira, H. H.; Vecchi, R.; Curi, R.; Calder, P. C.; Fernandes, L. C. Lifelong exposure to dietary fish oil alters macrophage responses in Walker 256 tumor-bearing rats. **Cell Imunology**, v. 231, n. 1-2, p. 56-62, 2004

Breitmeier, E. Terpenes: Importance, General Structure, and Biosynthesis. In: BREITMEIER, E. Terpenes: Flavors, Fragrances, Pharmaca, Pheromones. Berlin: Willey-VCH, 2006, p. 1-9.

Burdock, G. A. **Fenaroli's Handbook of Flavor Ingredients**, 6 ed. Boca Raton: CRC Press, 2010.

Calich, V.; Vaz, C. Imunologia. **2º ed. Imunologia**. Rio de Janeiro: Revinter, 2009, 323 p

Calvo, A. M.; Wilson, R. A.; Bok, J. W.; Keller, N. P. Relationship between secondary metabolism and fungal development. **Microbiology and Molecular Biology Reviews**. v. 66, p.447-459, 2002.

Cartum, J. Neuroblastoma: o enigmático tumor da infância. **Pediatria Moderna**, v. 48, p. 296-301, 2012.

Carvalho, C. C. R.; Fonseca, M. M. R. Towards the bio-production of trans-carveol and carvone form limonene: induction after cell growth on limonen and toluene. **Tetrahedron: Asymmetry**, v. 14, n.5, p.3925-3931, 2003.

Carvalho, C. C. R. de; Fonseca, M. M. R. da. Biotransformation of terpenes. **Biotechnology Advances**, v. 24, n. 2, p.134–142, 2006.

Carvalho, C. C. R. de.; Keulen, F. V.; Fonseca, M. M. R. da. Biotransformation of Limonene-1,2-epoxide to Limonene-1,2-diol by Rhodococcus erythropolis Cells An Introductory Approach to Selective Hydrolysis and Product Separation. **Food Technology and Biotechnology**, v. 38, n. 3, p.181-185, 2000.

Cathcart, M. K. Regulation of Superoxide Anion Production by NADPH Oxidase in Monocytes /Macrophages: Contributions to Atherosclerosis. **Arteriosclerosis, Thrombosis, and Vascular Biology**, v. 24, p. 23-28, 2003.

Chapla, V. M. Estudo químico e biológico do fungo endofítico *Phomopsis* sp. isolado da Senna spectabilis. Dissertação (mestrado em Química). Universidade Estadual Paulista, Araraquara, 2010

Chen, H. Modern solid state fermentation. New York: Springer, 2013.

Chen, Z.; Chu, H.; Chyau, C.; Chu, C.; Duh, P. Protective effects of sweet orange (Citrus sinensis) peel and their bioactive compounds on oxidative stress. **Food Chemistry**, v. 135, p. 2119–2127, 2012.

Chi, D. S.; Qui, M.; Gu, Krishnaswamya. Li, C.; Stone, W. Regulation of nitric oxide production from macrophages by lipopolysaccharide and catecholamines. **Nitric Oxide**, v. 8, n. 2, p. 127–132, 2003.

Cho, M.; Hunt, T. K.; Hussain, M. Z. Hydrogen peroxide stimulates macrophage vascular endothelial growth factor release. **American Journal of Physiology - Heart and Circulatory Physiology**, v. 280, p. 2357-2363, 2001.

Corrêa, R. C. G.; Rhoden, A. S.; Mota, T. R.; Azevedo, J. L.; Pamphile, J. A. Souza, C. G. M.; Polzeli, M. L. T. M.; Bracht, A.; Peralta, R. M. Endophytic fungi: expanding the arsenal of industrial enzyme producers. **Journal of Industrial Microbiology & Biotechnology**, v. 41, p.1467-1478, 2014.

Costa, J. L.; A.L L. Silva, A. L. L.; Bier, M. C. J.; Brondani, G. E.; Gollo, A. L.; Letti, L. A. J.; Erasmo, E. A. L.; Soccol, C. R. Callus Growth Kinetics of Physic Nut (Jatropha curcas L.) and Content of Fatty Acids from Crude Oil Obtained In Vitro. **Applied Biochemistry and Biotechnology**, v. 176, p. 892-902, 2015.

Crizel, T. M.; Jablonski, A.; Rios, A. O.; Rech, R.; Flôres, S. H. Dietary fiber from orange byproducts as a potential fat replacer. **Food Science and Technology**, v. 53, p. 9–14, 2013.

Crowell, P. L.; Elson. C. E.; Bailey. H. H.; Elegbede, A.; Haag, J. D.; Gould, M. N. Human metabolism of the experimental cancer therapeutic agent d-limonene. **Cancer Chemotherapy and Pharmacology**, v. 35, p. 31-37, 1994.

Cunico, M. M.; Miguel, O. G.; Miguel, M. D.; Carvalho, J. L. S.; Montrucchio, D. P.; Ferreira. J. L.; Oliveira, J. S. Extração de esteróides em frutos de Ottonia martiana MIQ., Piperaceae, com Gás Liquefeito. **Química Nova**, v. 26, p. 803-806, 2003.

da Costa, J. P.; Carvalhais, V.; Amado, F.; Silva, A.; Nogueira-Ferreira, R.; Ferreira, R. Helguero, L.; Vitorino, R. Anti-tumoral activity of human salivary peptides. **Peptides**, v. 71 p. 170-178, 2015.

Danielski, L.; Brunner, G.; Schwänke, C.; Zetzl, C.; Hense, H.; Donoso, J. P. M. Deterpenation of mandarin (Citrus reticulata) peel oils by means of countercurrent multistage extraction and adsorption/desorption with supercritical CO2. **Journal of Supercritical Fluids**, v. 44, p. 215-324, 2008.

Das, R., K.; Brar, S. K.; Verma, M. Potential use of pulp and paper solid waste for the bio-production of fumaric acid through submerged and solid state fermentation. **Journal of Cleaner Production**, v. 112, p. 4435-4444, 2016.

Dave, V.; Khirwadkar, P.; Dashora, K, 2014. A review on biotransformation. **Indian Journal of Research in Pharmacy and Biotechnology**, v. 2, p.1136-1140, 2014.

Deepika, V. B.; Murali, T. S.; Satyamoorthy, K. Modulation of genetic clusters for synthesis of bioactive molecules in fungal endophytes: A review. **Microbiological Research**, v. 182, p. 125–140, 2016.

Demyttenaere, J. C. R.; Kristof Van Belleghem, K. V.; De Kimpe, N. Biotransformation of (R)-(+)- and (S)-(−)-limonene by fungi and the use of solid phase microextraction for screening. **Phytochemistry**, v. 57, n. 2, p. 199–208, 2001.

Demyttenaere, J. C. R.; Belleghem, K. V.; De Kimpe, N. Biotransformation of (R)-(+)- and (S)-(-)-limonene by fungi and the use of solid phase microextraction for screening. **Phytochemistry**, v. 57, p.199-208, 2001.

Demyttenaere, J. C. R.; Belleghem, K.V.; De Kimpe, N. Biotransformation of terpenes by fungi: Study of the pathways involved. **Journal of Molecular Catalysis B: Enzymatic,** v. 11, p. 265–270, 2001.

Demyttenaere, J. C. R.; Vanorschelde, J.; Kimpe, N. Biotransformation of (R)-(+)- and (S)-(−)-citronellol by Aspergillus sp. and Penicillium sp., and the use of solid-phase microextraction for screening. **Journal of Chromatography**, v. 27, p.137-146, 2004.

Diaz, S.; Espinosa, S.; Brignole, E. A. Citrus peel oil deterpenation with supercritical fluids optimal process and solvent cycle design. **Journal of Supercritical Fluids**, v.35, p. 49-61, 2005.

Döll-Boscardin, P. M.; Sartoratto, A. Maia, B. H. L. N. S.; Paula, J. P. de, Nakashima, T.; Farago, P. V.; Kanunfre, C. C. In Vitro Cytotoxic Potential of Essential Oils of Eucalyptus benthamii and Its Related Terpenes on Tumor Cell Lines. **Evidence-Based Complementary and Alternative Medicine**, v. 2012 p. 1-8, 2012.

Duetz, W. A.; Fjällman, A. H. M.; Ren, S. Jourdat, C.; Witholt, B. Biotransformation of D-Limonene to (+)trans-Carveol by Toluene-Grown Rhodococcus opacus PWD4 Cells. **Applied and Environmental Microbiology**, v. 67, n. 6, p. 2829-2832, 2001.

Ďuračková, Z. Free Radicals and Antioxidants for Non-Experts. In: LAHER, I. Systems **Biology of Free Radicals and Antioxidants**. Berlin: Springer Berlin Heidelberg, 2014, p. 3-38.

Echeverría, G.; Graell, J.; Lara, I.; López, M. L. Physicochemical measurements in 'Mondial Gala®' apples stored at different atmospheres: Influence on consumer acceptability. **Postharvest Biology and Technology**, v. 50, p. 135-144, 2008.

El-Akhal, F.; Lalami, A. E.; O. Guemmouh, R. Larvicidal activity of essential oils of Citrus sinensis and Citrus aurantium (Rutaceae) cultivated in Morocco against the malaria vector Anopheles labranchiae (Diptera: Culicidae). **Asian Pacific Journal of Tropical Disease**, v. 5, p. 458-462, 2015.

Ezeonu, F. C.; Chidume, G. I.; Udedi, S. C. Insecticidal properties of volatile extracts of orange peels. **Bioresource Technology**, v. 76, p. 273-274, 2001.

FDA. Limonene Glycol. Acesso em: 05/12/2015. Disponível em: http://www.accessdata.fda.gov/scripts/fcn/fcnNavigation.cfm?filter=1946-00-5&sortColumn=&rpt=eafusListing

Ferreira, M. C.; Vieira, M. L. A.; Zani, C. L.; Alves, T. M. A.; Junior, P. A. S.; Murta, S. M. F.; Romanha, A.J.; Gil, L. H. V. G.; Carvalho, A. G. O.; Zilli, J. E.; Vital, M. J. S.; Rosa, C. A.; Rosa, L. H. Molecular phylogeny, diversity, symbiosis and discover of bioactive compounds of endophytic fungi associated with the medicinal Amazonian plant *Carapa guianensis* Aublet (Meliaceae). **Biochemical Systematics and Ecology**, v. 59, p.36-44, 2015.

Fiorentin, L .D.; Menon, B. T.; de Barros, S. T. D.; Pereira, N. C.; Lima, O. C. M.; Modenes, A. N. Isotermas de sorção do resíduo agroindustrial bagaço de laranja. **Revista Brasileira de Engenharia Agrícola e Ambiental**, v. 14, p. 653–659, 2010.

Fischer, J. S. G.; Mota e Silva, M. S.; Paschoal, M.; Carvalho, P. C.; Carvalho, M. G. C. Efeito do álcool perílico na expressão gênica de células de adenocarcinoma de pulmão humano. **Jornal Brasileiro de Pneumologia**, v. 31, p. 511-515, 2015.

Fisher, K.; Phillips, C. Potential antimicrobial uses of essential oils in food: is citrus the answer?. **Trends in Food Science & Technology**, v. 9, p.156-164, 2008.

Fouda, A. H.; Hassan, S. E-D.; Eid, A. M.; Ewais, E. E-D. Biotechnological applications of fungal endophytes associated with medicinal plant Asclepias sinaica (Bioss.). **Annals of Agricultural Sciences**, v. 60, n. 1, p. 95–104, 2015.

Gad, S. C. **Development of Therapeutic Agents Handbook**. New Jersey: Wiley, 2011.

Gautam, N.; Mantha, A. K.; Mitall, S. Essential Oils and Their Constituents as Anticancer Agents: A Mechanistic View. **BioMed Research Internationa**, v. 2014, 23 p., 2014.

Gea-Bancloche, J. C. Immunomodulation. In: RUNGE, M. S. PATTERSON, C. **Principles of Molecular Medicine**. Nova Iorque: Springer Publishing, 2006, p. 893-904.

Gelb, M.; Tamanol, F.; Yokoyama, K.; Esson, K.; Gould, M. N. The inhibition of protein prenyltransferases by oxygenated metabolites of limonene and perillyl alcohol. **Cancer Letters**, v. 91, p.169-175, 1995.

Gollo, A. L.; Silva, A. L. L. de, Lima, K. K. D.; de. Costa, J. L.; Camara, M. C.; Biasi, L. A.; Rodrigues, C.; Vandenberghe, L. P. S.; Soccol, V. T.; Soccol, C. R. Developing a Plant Culture Medium Composed of Vinasse Originating from *Haematoccus pluvialis* Culture. **Pakistan Journal of Botany**, v. 48, n.1, p. 295-303, 2016.

Gomes, P. B.; Mata, V. G.; Rodrigues, A. E. Production of rose geranium oil using supercritical fluid extraction. **Journal of Supercritical Fluids,** v. 41, p. 50-60, 2007.

Gosslau A.; Chen, K. Y.; Ho, C.; Li, S. Anti-inflammatory effects of characterized orange peel extracts enriched with bioactive polymethoxyflavones, **Food Science and Human Wellness**, v. 3, p. 26-35, 2014.

Guo, X. M.; Lu, Q.; Liu Z. J.; Wang, L. F.; Feng, B. A.; Effects of D-limonene on leukemia cells HL-60 and K562 in vitro. **Journal of Experimental Hematology**, v. 14, n. 4, p. 692-695, 2006.

Gupta, S.; Pramanik, S.; Tiwari, O.; Thacker, N.; Pande, M.; Upmanyu, N. Immunomodulatory activity of gymnema sylvestre leaves. **The Internet Journal of Pharmacology**, v. 8, n. 2, 2010.

Hassan, S. B.; Gali-Muhtasib, H.; Goransson, H.; Larsson, R. Alpha Terpineol: A Potential Anticancer Agent which Acts through Suppressing NF-κB Signalling. **Anticancer Researches**, v. 30, p. 1911-1919, 2010.

Heath, H. B. **Source Book of Flavors: AVI Sourcebook and Handbook Series**. New York: Van Nostrand Reinhold, 1981.

Houjin, L.; Wenjian, L.; Chuanghua, C.; Yipin, Z.; Yongcheng, L. 2006. Biotransformation of limonene by marine bacteria. **Chinese Journal of Analytical Chemistry**, v. 34, p. 946-950, 2006.

Hu, J. New Developments in terpenes research. Columbus: Nova Science Pub, 2013.

Huang, D.; Ou, B.; Hampsch-Woodill, M.; Flanagan, J.;Prior, R. High-throughput Assay of Oxygen Radical Absorbance Capacity (ORAC) Using a Multichannel Liquid Handling System Coupled with a Microplate Fluorescence Reader in 96-Well Format. **Journal of Agriculture and Food Chemistry**, v. 50, p. 4437-4444, 2002.

Hue, C.; Gunata, Z.; Bergounhou, A.; Assemat, S.; Boulanger, R.; Sauvage, F. X; Davrieux, F. (2014) Near infrared spectroscopy as a new tool to determine cocoa fermentation levels through ammonia nitrogen quantification. **Food Chemistry**, v. 148, p. 240-245, 2014.

Instituto Adolfo Lutz. **Normas Analíticas do Instituto Adolfo Lutz** (Vol 1: Métodos Físico-Químicos para Análise de Alimentos), 4th ed. São Paulo: Instituto Adolfo Lutz, 2008.

Isaka, M.; Palasarn, S.; Kocharin, K.; Hywel-Jones, N. L. Comparison of the bioactive secondary metabolites from the scale insect pathogens, anamorph *Paecilomyces* cinnamomeus, and teleomorph *Torrubiella luteorostrata*. **Journal of Antibiotics**, v. 60, p. 577–581, 2007.

Jaafari, A.; Tilaoui, M.; Mouse, H. A.; M'bark, L. A.; Aboufatima, R.; Chait, A. Lepoivre, M.; Zyad, A. Comparative study of the antitumor effect of natural

monoterpenes: relationship to cell cycle analysis. **Brazilian Journal of Pharmacognosy**, v. 22, n. 3, p. 534-540, 2012.

Janeway, C. A.; Travers, P.; Walport T. M.; Capra, J.D. **Imunobiologia. O sistema imunológico na saúde e na doença**. 5ª ed. Porto Alegre: ArtMed, 2002.

Jayaprakasha, G. K.; Girennavar, B.; and Pati, B. S. Antioxidant capacity of pummelo and navel oranges: Extraction efficiency of solvents in sequence. **LWT – Food Science and Technlogy**, v. 41, p. 376-384, 2008.

Jing, L.; Zhang, Y.; Fan, S.; Gu, M.; Guan, Y.; Lu, X.; Huang, C.; Zhou, Z. Preventive and ameliorating effects of citrus D-limonene on dyslipidemia and hyperglycemia in mice with high-fat diet-induced obesity. **European Journal of Pharmacology**, v. 715, p. 46–55, 2013.

Kaur, J.; Kaur, G. An insight into the role of citrus bioactives in modulation of colon cancer. **Journal of Functional Foods**, v. 13, p. 239-261, 2015.

Kofujita, H.; Ettyu, K.; Ota, M. Characterization of the major components in bark from five Japanese tree species for chemical utilization. **Wood Science and Technology**, v. 33, p. 223-228, 1999.

Krassimir, D. N.; Tremblav, F. M.; Fenton, N. J.; Alexandrov, A. Structure of Pinus nigra Arn. populations in Bulgaria revealed by chloroplast microsatellites and terpenes analysis: Provenance tests. **Biochemical Systematics and Ecology**, v. 34, p.562-574, 2006.

Ku, C. S.; Sathishkumar, M.; Mun, S. P. Binding affinity of proanthocyanidin from waste Pinus radiate bark onto proline-rich bovine Achilles tendon collagen type I. **Chemosphere**, v. 67, p. 1618-1627, 2007

Kupcinskiene, E.; Stikliene, A.; Judzentiene, A. The essential oil qualitative and quantitative composition in the needles of Pinus sylvestris L. growing along industrial transects. **Environmental Pollution**, v. 155, p. 481-491, 2008.

Kusari, S. Singh, S. Jayabaskaran, C. Biotechnological potential of plant-associated endophytic fungi: hope versus hype, Trends in Biotechnology 32 (6) (2014) 297-303

Lebovka, N.; Vorobiev, E.; Chemat, F. **Enhancing Extraction Processes in the Food Industry**. Boca Raton: CRC Press, 2011.

Lima, N. G. P. B.; Sousa, D. P.; de, Pimenta, F. C. F.; Alvez, M. F.; Souza, F. S. de; Macedo, R. O.; Cardoso, R. B.; Morais, L. C. S. L. de; Diniz, M. F. F. M.; Almeida, R.

N. de. Anxiolytic-like activity and GC–MS analysis of (R)-(+)-limonene fragrance, a natural compound found in foods and plants. **Pharmacology, Biochemistry and Behavior**, v.103, p. 450-454, 2013.

Liu, K.; Chen, Q.; Liu, Y.; Zhou, X.; Wang, X. Isolation and biological activities of decanal, linalool. valencene, and octanal from sweet orange oil. **Journal of Food Science,** v. 77, p. 1156-1161, 2012.

Liu, X.; Dong, M.; Chen, X.; Jiang, M.; Lu, X.; Yan, G. Antioxidant activity and phenolics of an endophytic Xylaria sp. from *Ginkgo biloba*. **Food Chemistry**, v. 105, n. 2, p. 548-554 , 2007.

Logarto, P. A.; Silva, Y. R.; Guerra, S. I.; Iglesias, B. L. Comparative study of the assay of Artemia salina L. and the estimate of the medium lethal dose (LD50 value) in mice to determine oral acute toxicity of plant extracts. **Phytomedicine**, v. 8, n. 5, p. 395-400, 2001.

Lopresto, C. G.; Petrillo, F.; Casazza, A. A.; Aliakbarian, B.; Perego, P.; Calabro, V. A non-conventional method to extract D-limonene from waste lemon peels and comparison with traditional Soxhlet extraction. **Separation and Purification Technology**, v. 137, p. 13-20, 2014.

Lu, Y.; Zhao, S.; Zhang, J.; Zhang, H.; Xie, Z.; Cai, G.; Jiang, W. Effect of orange peel essential oil on oxidative stress in AOM animals. **International Journal of Biological Macromolecules**, v. 50, p.1144-1150, 2012.

Ma, Y-Q.; Chen, J-C.; Liu, D-H.; Ye, X-Q.) Effect of Ultrasonic Treatment on the Total Phenolic and Antioxidant Activity of Extracts from Citrus Peel. **Journal of Food Science**, v. 73, p. 115-120., 2008.

MacMicking J.; Xie Q. W.; Nathan C. Nitric oxide and macrophage function. **Annual Review of Immunology**, v. 15, p. 323-350, 1997.

Madhavi, D. L.; Deshpande, S. S.; Salunkhe, D. K. Food Antioxidants: Technological. **Toxicological and Health Perspectives**. New York: Marcel Dekker, 1996.

Mahmood, A.U.; Greenman, J.; Scragg, A. H. Orange and potato peel extracts: Analysis and use as Bacillus substrates for the production of extracellular enzymes in continuous culture. **Enzyme and Microbial Technology**, v. 22, p. 130-137, 1998.

Malhotra, S.; Suri, S.; Tuli, R. Antioxidant activity of citrus cultivars and chemical composition of *Citrus karna* essential oil. **Planta Médica**, v. 75, p. 62-44, 2009.

Mantzouridou, F. T.; Paraskevopoulou, A.; Lalou, S. Yeast flavour production by solid state fermentation of orange peel Waste. **Biochemical Engineering Journal**, v. 101, p.1–8, 2005.

Maróstica Jr, M. R.; Pastore, G. M.; Production of R-(+)-α-Terpineol by the biotransformation of limonene from orange essential oil, using cassava waste water as médium. **Food Chemistry**. v. 101, n. 1, p. 345-350, 2007.

Maróstica Junior, M. R.; Rocha e Silva, T. A. A.; Franchi, G. C.; Nowill, A.; Pastore, G. M.; Hyslop, S. Antioxidant potential of aroma compounds obtained by limonene biotransformation of orange essential oil. **Food Chemistry**, v. 116, p. 8–12, 2009.

Maróstica Júnior, M. R.; Pastore, G. M. Biotransformation of limonene: a review of the main metabolic pathways. **Química Nova**, v. 30, n. 2, p. 382-387, 2007.

Maróstica Júnior, M. R.; Silva, T. A. A. R.; Franchi, G. C.; Nowill, A.; Pastore, G. M.; Hyslop, S. Antioxidant potential of aroma compounds obtained by limonene biotransformation of orange essential oil. **Food Chemistry**, v. 116, p. 8-12, 2009.

Matsumae, A.; Kamio, Y.; Hata, T. Studies on cerulenin producing strain. **Journal of Antibiotics**, v. 16, p. 239-243, 1963.

Mcgraw, G. W.; Hemingway, R. W.; Ingram Jr., L. L.; Canady, C. S.; Macgraw, W. B.Thermal Degradation of Terpenes: Camphene, Δ^3-Carene, Limonene, and α-Terpinene. **Environmental Science Technology,** v. 33, p. 4029-4033, 1999.

Miller, J. A.; Thompson, P. A.; Hakim, I. A.; Chow, H. S.; Thomson, C. A. D-Limonene: a bioactive food component from citrus and evidence for a potential role in breast cancer prevention and treatment. **Oncology Review**, v. 5, p. 31-42, 2011.

Molina, G.; Pastore, G. M. Biotransformation of Monoterpenes by Endophytes Isolated From Brazilian Fruits. International Proceedings of Chemical. **Biological & Environmental**, v. 50, p. 59-64, 2013.

Molina, G.; Bution, M. L.; Bicas, J. L.; Dolder, M. A. H.; Pastore, G. M. Comparative study of the bioconversion process using R-(+)- and S-(–)-limonene as substrates for Fusarium oxysporum 152B. **Food Chemistry**, v. 174, p. 606–613, 2015.

Molina, G.; Pimentel, M. R.; Bertucci, T. C. P.; Pastore, G. M. Application of fungal endophytes in biotechnological processes. **Chemical Engineering Transactions**, v. 27, p. 289-294, 2012.

Morath, S. U.; Hung, R.; Bennet, J. W. Fungal volatile organic compounds: A review with emphasis on their biotechnological potential. **Fungal biology reviews**, v. 26, p.73-83, 2012.

Morcia, C.; Tumino, G.; Ghizzoni, R.; Terzi, V. Carvone (Mentha spicata L.) Oils. In: PREEDY, V. **Elsevier Essential Oils in Food Preservation**, **Flavor and Safety,** Boston: Elsevier, 2015, p. 309-316.

Mordor Intelligence LLP, Global Flavor and Fragrance Industry 2016 Market Research Report. Disponível em: http://www.reportlinker.com/p03024563/Brazil-Food-Flavor-and-Enhancer-market-Growth-Trends-Forecast-for-the-period.html

Moslovec, T; Jokić, S.; Subarić, D.; Aladić, k.; Vukoja, J.; Prce, N. Pressing and supercritical CO2 extraction of Camelina sativa oil. **Industrial Crops and Products,** v. 54, p. 122-129, 2014.

Mousa, W. K.; Raizada, M. N. The Diversity of Anti-Microbial Secondary Metabolites Produced by Fungal Endophytes: An Interdisciplinary Perspective. **Frontiers in Microbiology**, v. 4, p. 1-18, 2013.

Mueller, G. M.; Bills, G. F.; Foster, M. S. **Biodiversity of Fungi: Inventory and Monitoring Methods**. Boston: Elsevier Academic Press, 2014.

Mukherjee, B. B.; Kraidman, G.; Hill, I. D. Synthesis of Glycols by Microbial Transformation of Some Monocyclic Terpenes. **Applied Microbiology**, v. 25, n. 3, p. 447-453, 1973.

Murray, M. T. **The Complete Book of Juicing, Revised and Updated: Your Delicious Guide to Youthful Vitality**. New York: Clarkson Potter. 2013.

Murthy, K. N. C.; Jayaprakasha, G. K.; Patil, B. S. D-limonene rich volatile oil from blood oranges inhibits angiogenesis, metastasis and cell death in human colon cancer cells. **Life Sciences**, v. 91, p. 429-439, 2012.

Nakatani, N.. Phenolic antioxidants from herbs and spices. **Biofactors**, v. 13, p. 141–146, 2000.

Netto, B. **Press Release: Análise de Dados de Estimativa de Safra 2015/16**. Araraquara: CitrusBR – Assossiação nacional dos exportadores de sucos cítricos, 2015

Nguta, J. M.; Mbaria, J. M.; Gakuya, D. W.; Gathumbi, P. K.; Kabasa, J. D.; Kiama, S. G.; Evaluation of Acute Toxicity of Crude Plant Extracts from Kenyan Biodiversity, using Brine Shrimp, Artemia salina L. (Artemiidae). **The Open Conference Proceedings Journal**, v. 3, p. 30-34, 2012.

Nicolini, L.; Hunolstein, C. V.; Carilli, A. Solid state fermentation of orange peel and grape stalks by Pleurotus ostreatus, Agrocybe aegerita, and Armillariella mellea. **Applied Microbiology and Biotechnology**, v. 26, n. 1, p.95-98, 1987.

NIST 14 Mass Spectral Library & Search Software v.2.2 (2014-04) (NIST 2014/EPA/NIH). Gaithersburg: National Institute of Standards and Technology, 2014.

NIST 98 Library with Search Software (Version 1.7). Gaithersburg: National institute of standards and technology, 1999.

Nithya, K.; Muthumary, J.; Secondary Metabolite from *Phomopsis sp.* isolated from *Plumeria acutifolia* Poiret. Recent Research in Science and Technology, v. 2, p. 99-103, 2010.

Nogueira, A.; Santos, L. D.; Paganini, C.; Wosiacki, G. Avaliação da fermentação alcoólica do extrato de bagaço de maçã. **Semina: Ciências Agrárias**, v. 26, p.187-194, 2005.

NPCS Board of Consultants & Engineers. **The Complete Technology Book on Alcoholic and Non-Alcoholic Beverages (Fruit Juices, Whisky, Beer, Rum and Wine).** Delhi: Asia Pacific Business Press Inc, 2008.

Olano, C.; Méndez, C.; Salas, J. A.; Strategies for the Design and Discovery of Novel Antibiotics using Genetic Engineering and Genome Mining. In: VILLA, T. G. VEIGA-CRESPO, P. **Antimicrobial Compounds. Berlin: Springer Berlin Heidelberg**, 2013, p. 1-25.
Oliveira, J. S Extrator para Operação com Gases Liquefeitos, Brazilian Patent PI 9804199-1, May 23, 2000.

Omura, S. The antibiotic cerulenin, a novel tool for biochemistry as an inhibitor of fatty acid synthesis. **Bacteriological Reviews**, v. 40, p. 681–697, 1976.

Ordoudi, S. A.; Mantzouridou, F.; Daftsiou, E.; Malo, C.; Hatzidimitriou, E.; Nenadis, N. and Tsimidou, M. Z. (2014) Pomegranate juice functional constituents after alcoholic and acetic acid fermentation. **Journal of Functional Foods**, v. 8, p. 161-168, 2014.

Orlandelli, R. C.; Silva, M. L. C.; da. Vasconcelos, A. F. D.; Almdeira, I. V.; Vicentini, V. E. P.; Prieto, A.; Hernandez, M. D. D.; Azevedo, J. L.; Pamphilea, J. A. (1→3,1→6)-

d-glucans produced by *Diaporthe sp*. endophytes: Purification, chemical characterization and antiproliferative activity against MCF-7 and HepG2-C3A cells. **International Journal of Biological Macromolecules**, v. 94, p. 431-437, 2017.

Orlandelli, R. C.; Vasconcelos, A. F. B.; Azevedo, J. L.; Silva, M. L. C.; da. Pamphile, J. A. Screening of endophytic sources of exopolysaccharides: Preliminary characterization of crude exopolysaccharide produced by submerged culture of *Diaporthe sp*. JF766998 under different cultivation time. **Biochimie Open**, v. 2, p. 33-40, 2016.

Oses, R.; Valenzuela, S.; Freer, J.; Baeza, J.; Rodríguez, J. Evaluation of fungal endophytes for lignocellulolytic enzyme production and wood biodegradation. **International Biodeterioration & Biodegradation**, v. 57, n. 2, p.129-135, 2016.

Pandey, A. Solid state fermentation. **Biochemical Engineering Journal**, v. 13, p. 81–88, 2003.

Pandey, A.; Soccol, C. R.; Larroche, C. Production of aroma compounds**.** In: PANDEY, A.; FERNANDES, M.; LARROCHE, C. **Current developments in Solid-state Fermentation**. New Delhi, New York: Asiatech publishers Inc, Springer, 2008.

Parashar, S.; Sharma, H.; Garg, M. Antimicrobial and Antioxidant activities of fruits and vegetable peels: A review. **Journal of Pharmacognosy and Phytochemistry**, v. 3, p. 160-164, 2014.

Pérez-Mosqueda, L. M.; Trujillo-Cayado, L. A.; Carrillo, F.; Ramirez, P.; Muñoz, J.; Formulation and optimization by experimental design of eco-friendly emulsions based on D-limonene. **Colloids and Surfaces B: Biointerfaces**, v. 128, p.127-131, 2015.

Pick E.; Mizel D. Rapid microassays for the measurement of superoxide and hydrogen peroxide production by macrophages in culture using an automatic enzyme immunoassay reader. **Journal of Immunology Methods**, v. 46, p. 211-226, 1981.

Pimentel, M. R.; Molina, G.; Dionísio, A. P.; Maróstica Junior, M. R.; Pastore, G. M. The use of endophytes to obtain bioactive compounds and their application in biotransformation process. **Biotechnology Research International**, v. 2011, p.1-11, 2011.

Poon, G. K.; Vigushin, D.; Griggs, L. J.; Rowlands, M. G.; Coombes, R. C.; Jarman, M. Identification and characterization of limonene metabolites in patients with advanced cancer by liquid chromatography/mass spectrometry. **Drug Metabolism & Disposition**, v. 24, n. 5, p. 565-571, 1996.

Pourbafrani, M.; Forgács, G.; Horváth, I. S.; Niklasson, C.; Taherzadeh M. J. Production of biofuels, limonene and pectin from citrus wastes Bioresource. **Technology**, v. 101, n. 11, p. 4246-4250, 2010.

Qadri, M.; Deshidi, R.; Shah, B. A.; Bindu, K.; Vishwakarma, R. A.; Riyaz-Ul-Hassan, S. 2015. An endophyte of *Picrorhiza kurroa* Royle ex. Benth, producing menthol, phenylethyl alcohol and 3-hydroxypropionic acid, and other volatile organic compounds. **World Journal of Microbiology and Biotechnology**, v. 31, p. 1647-1654, 2015.

Rakshith, D.; Santosh, P.; Satisha, S.; Isolation and characterization of antimicrobial metabolite producing endophytic *Phomopsis* sp. from Ficus pumila Linn. (Moraceae), **International Journal of Chemical and Analytical Science**, 4, n. 3, p. 156–160, 2013.

Reda, S. Y.; Leal, E. S.; Batista, A. C.; Barana, A. C.; Schinitzel, E. S.; Carneiros, P. I. B. Caracterização dos Óleos das Sementes de Limão Rosa (*Citrus limonia* Osbeck) e limão siciliano (*Citrus limon*), um Resíduo Agroindustrial. **Ciência e Tecnologia de Alimentos**, v. 25, p. 672-676, 2005.

Reineccius, G. **Sourcebook of Flavors**. 2nd edition. New York: Springer, 1998.

Rezzadori, K.; Beneditti, S.; Amante, E. R. Proposals for the residues recovery: orange waste as raw material for new products. **Food Bioprod Process**, v. 90, n. 4, p. 606-614. 2012.

Roberto, D.; Micucci, P.; Sebastian, T.; Graciela, F.; Anesini, C. Antioxidant Activity of Limonene on Normal Murine Lymphocytes: Relation to H_2O_2 Modulation and Cell Proliferation. **Basic & Clinical Pharmacology & Toxicology**, v. 106, p. 38-44, 2009.

Rodriguez, R. J.; White Jr, J. F.; Arnold, A. E.; Redman, R. S.; Fungal endophytes: diversity and functional roles. **New Phytologist**, v.182, n. 2, p. 314-330, 2009.

Rottava, I.; Cortina, P. F.; Martello, E.; Cansian, R. L.; Toniazzo, G.; Antunes, O. A.; Oestreicher, E. G.; Treichel, H.; de Oliveira, D. Optimization of α-Terpineol production by the biotransformation of R-(+)-limonene and (-)-β-pinene. **Applied Biochemistry and Biotechnology**, v. 164, n. 4, p. 514-523, 2011.

Rubel, R.; Santa, H. D. S.; Fernandes, L. C.; Bonatto, S. J. R.; Bello, S.; Figueiredo, B. C.; Lima Filho, J. H.; Santos, C. A. M.; Soccol, C. R. Hypolipidemic and antioxidant properties of Ganoderma lucidum (Leyss:Fr) Karst used as a dietary supplement. **World Journal of Microbiology and Biotechnology**, v. 27, p.1083-1089, 2011.

Rubel, R.; Santa, H. S. D.; Bonatto, S. J. R.; Bello, S.; Fernandes, L. C.; Bernardi, R. D.; Gern, J.; Santos, C. A.; Soccol, C. R. Medicinal Mushroom Ganoderma lucidum (Leyss: Fr) Karst. Triggers Immunomodulatory Effects and Reduces Nitric Oxide Synthesis in Mice. **Journal of Medicinal Food,** v.13, p. 142-148, 2010.

Rueda, A. Q.; Rodriguez, I. G.; Arantes, E. C.; Setúbal, S. S.; Calderon, L. A.; Zuliani, J. P.; Stábeli, R. G.; Soares, A. M. Biochemical Characterization, Action on Macrophages, and Superoxide Anion Production of Four Basic Phospholipases A2 from Panamanian Bothrops asper Snake Venom. **Biomedical Research International**, v. 2013, 9 p., 2013.

Rukachaisirikul, V.; Sommart, U.; Phongpaichit, S.; Sakayaroj, J.; Kirtikara, K. Metabolites from the endophytic fungus *Phomopsis* sp. PSU-D15. **Phytochemistry**, v. 69, n. 3, p. 783-787, 2008.

Samson, R. A.; Pitt, J. I. **Modern Concepts in *Penicillium* and *Aspergillus* classification**. 1st ed. New York: Springer Science Business Media, 1990.

Santi, G.; Crognale, S.; D'Annibale, A.; Petruccioli, M.; Ruzzi, M.; Valentini, R.; Moresi M. Orange peel pretreatment in a novel lab-scale direct steam-injection apparatus for ethanol production. **Biomass and Bioenergy**, v. 61, p. 146–156, 2014.

Santos, L. F. dos. Produção e Aplicação da levana de *Bacillus subtilis* natto em filmes isolados para liberação em cólon – específica de fármacos. 255 f. Dissertação (Mestrado em Biotecnologia) – Área agroindustrial, Universidade Estadual de Londrina, Londrina, 2010.

Sarrou, E.; Chatzopoulou, P.; Dimassi-Theriou, K.; Therios, L. Volatile Constituents and Antioxidant Activity of Peel, Flowers and Leaf Oils of *Citrus aurantium* L. Growing in Greece. **Molecules**, v. 18, p. 10639-10647, 2013.

Schepetkin, I A.; Quinn, M. T. Botanical polysaccharides: macrophage immunomodulation and therapeutic potential. **International immunopharmacology**, v. 6, p. 317-333, 2016.

Schmidt, C. G.; Furlong, E. B. Effect of particle size and ammonium sulfate concentration on rice bran fermentation with the fungus *Rhizopus oryzae.* **Bioresource Technology**, v. 123, p. 36–41, 2012

Selvi, M. T. Thirugnanasampandan, R. Sundarammal, S. Antioxidant and cytotoxic activities of essential oil of Ocimum canum Sims. from India. **Journal of Saudi Chemical Society**, v. 19, p. 97-100, 2015

Semiz, G.; Heijari, J.; Isiki, K.; Holopainen, J. K. Variation in needle terpenoids among Pinus sylvestris L. (Pinaceae) provenances from Turkey. **Biochemical Systematics and Ecology**, v. 35, p. 652-661, 2007.

Serra, S. Recent Developments in the Synthesis of the Flavors and Fragrances of Terpenoid Origin. In: RAHMAN, A-U. **Studies in Natural Products Chemistry**. Boston: Elsevier, v. 46, p. 201-226, 2015.

Shamtsyan, M.; Konusova, V.; Maksimova, Y.; Goloshchev, A.; Panchenko, A.; Simbirtsev, A.; Petrischev, N.; Denisova, N. Immunomodulating and anti-tumor action of extracts of several mushrooms. **Journal of biotechnology**, v. 113, p. 77-83, 2004.

Shashirekha, M. N.; Baskaran, R.; Jaganmohan, L. Influence of processing conditions on flavour compounds of custard apple (Annona squamosa L.). **LWT - Food Science and Technology**, v. 41, p. 236-243, 2008.

Shrader, J. Microbial Flavour Production. In: BERGER, R. G. Flavours and Fragrances: Chemistry, Bioprocessing and Sustainability. New York: Springer Berlin Heidelberg, 2004.

Silva, A. L .L.; Rodrigues. C.; Costa, J. L.; Machado, M. P.; Penha, R. O.; Biasi, L. A.; Vandenberghe, L. P. S.; Soccol, C.R. Gibberellic acid fermented extract obtained by solid-state fermentation using citric pulp by *Fusarium moniliforme*: Influence on *Lavandula angustifolia* Mill., cultivated *in vitro*. **Pakistan Journal of Botany,** v. 45, p. 2057-2064, 2013.

Silva, J. R. F.; Cantelli, K. C.; Soares, M. B. A.; Tres, M. V.; Oliveira, D.; Meireles, M. A.; Oliveira, J. V.; Treichel, H.; Mazutti, M. A. Enzymatic hydrolysis of non-treated sugarcane bagasse using pressurized liquefied petroleum gas with and without ultrasound assistance. **Renewable Energy**, v. 83, p. 674-679, 2015.

Silva, L. V.; Constâncio, S. M.; Mendes, M. F.; Coelho, G. L. V. Extração de Óleo Essencial da Pimenta Rosa (Schinus molle) usando Hidrodestilação e Soxhlet, in: Proceedings of VI Congresso Brasileiro de Engenharia Química em Iniciação Científica, Campinas, 2005.

Silva, W.D. Mota, I. **Bier imunologia básica e aplicada**. 5.ed. Rio de Janeiro: Guanabara Koogan, 2003.

Silvestein, R. M.; Bassler, G. C.; Morril, T. C. **Spectroscopic identification of organic compounds**. 5th ed. New Jersey: John Wiley & Sons, 1991.

Simon, J. A.; Szankasi, P.; Nguyen, D. K.; Ludlow, C.; Dunstan, H. M.; Roberts, C. J.; Jensen, E. L.; Hartwell, L. H.; Friend, S. H. Differential toxicities of anticancer agents among DNA repair and checkpoint mutants of Saccharomyces cerevisiae. **Cancer Research**, v. 60, n. 2, p. 328-333, 2000.

Singh, P.; Shukla, R.; Prakash, B.; Kumar A.; Singh, S.; Mishra, P. K.; Dubey, N. K. Chemical profile, antifungal, antiaflatoxigenic and antioxidant activity of Citrus maxima Burm. and Citrus sinensis (L.) Osbeck essential oils and their cyclic monoterpene, DL-limonene. **Food and Chemical Toxicology**, v. 48, p. 1734-1740, 2010.

Singhania, R. R.; Patel, A. K.; Soccol, C.; Pandey, A. Recent advances in solid-state fermentation. **Biochemical Engineering Journal**, v. 44, n. 1, p.13-18, 2008.

Singleton, V. L.; Rossi Jr, J. A Colorimetry of total phenolics with phospomolybdic–phospotungstic acid reagents. **American Journal of Enology and Viticulture**, v. 16, p**.** 144-158, 1965.

Smit, A. Y.; Engelbrecht, L.; Toit, M. Managing Your Wine Fermentation to Reduce the Risk of Biogenic Amine Formation. **Frontiers in Microbiology,** v. 3, p.1-10, 2012.

Soccol, C. R.; Medeiros, A. B. P.; Vanderberghe, L. P. S.; Soares, M.; Pandey, A. Production of Aroma Compounds. In: PANDEY, A. SOCCOL, C. R. LARROCHE, C. **Current Developments in Solid-state Fermentation**. New York: Springer, 2008.

Soccol, C.R., Medeiros, A.B.P., Bier, M.C.J. Bioprocesso para a produção de derivados de terpenos por *Diaporthe sp.* e *Rhodotorula mucilaginosa.* Patente INPI protocolo BR 102014029813, 28/11/2014.

Solis, P. N.; Wright, C. W.; Anderson, M. M.; Gupta, M. P.; Phillipson, J. D. A microwell cytotoxicity assay using *Artemia salina* (brine shrimp), **Planta Médica**, v.59, n. 3, p.250-252, 1993..

Somogyi, M. Estimation of sugars by colorimetric method. **Journal of Biological Chemistry 200**, v. 245, 1952.

Song, F-L.; Gan, R-Y.; Zhang, Y.; Xiao, Q.; Kuang, L.; Li, H-B. (2010) Total Phenolic Contents and Antioxidant Capacities of Selected Chinese Medicinal Plants, International. **Journal of Molecular Sciences**, v. 11, p. 2362-2372, 2010.

Sorenson, W. G.; Frazer, D. G.; Jarvis, B. B.; Simpson, J.; Robinson, V. A. 1987. Trichothecene mycotoxins in acrolized conidia of *Stachybotrys atra.* **Applied and Environmental Microbiology**, v. 53, p. 1370-1375, 1987.

Steinbrecher, R.; Hauff, K.; Riissler, J.; Diirr, M.; Seufert, G. Monoterpene Emission from Valencian Citrus Belt, Spain Soils in Orange Plantations of the Spain. **Physics and Chemistry of the Earth B**, v. 24, p. 695-698, 1999.

Stojković, M. B.; Mitić, S. S.; Pavlović, J. L.; Stojanović, B. T. Paunović, D. D. (2014) Antioxidant potential of *Tanacetum vulgare* L. extracts. **Biologica** Nyssana, v. 5, p. 47-51, 2014.

Strobel, D.; Daisy, B. Bioprospecting for Microbial Endophytes and Their Natural Products. **Microbiology and Molecular Biology Reviews**, v. 67, n. 4, p. 491-502, 2003.

Subramaniyam, R.; Vimala, R. Solid state and submerged fermentation for the production of bioactive substances: a comparative study. **International Journal of Science and Nature**, v. 3, p. 480-486, 2012.

Sun, X. Guo, L-D. Endophytic fungal diversity: review of traditional and molecular techniques**. Mycology: An International Journal on Fungal Biology**, v. 3, n. 1, p. 65-76, 2012.

Suryanarayanan, T. S.; Ravishankar, J. P.; Venkatesan, G.; Murali, T. S. Characterization of the melanin pigment of a cosmopolitan fungal endophyte. **Mycological Research**, v. 108, p. 974-978, 2004.

Tai, Y-N. ; Xu, M. ; Ren, J-N. ; Dong, M. ; Yang, Z-Y. ; Fan, G. Optimisation of α-terpineol production by limonene biotransformation using *Penicillium digitatum* DSM 62840. **Journal of Science and Food Agriculture,** v. 96, p. 965-961, 2015.

Tan, Q.; Day, D. F.; Cadwallader, K. R. Bioconversion of (R)-(+)-limonene by P. digitatum (NRRL 1202). **Process Biochemistry**. v. 33, n. 1, p. 29-31, 1998

Tang, D.; Shi, Y.; Kang, R.; Li. T.; Xiao, W.; Wang, H.; Xiao, X. Hydrogen peroxide stimulates macrophages and monocytes to actively release HMGB1. **Journal of Leukocyte Biology**, v. 81, p. 741-747, 2007.

Tiwari, P.; Mishra, N.; Sangwan, N. Phytochemical and Pharmacological Properties of *Gymnema sylvestre*: An Important Medicinal Plant. **BioMed Research International**. v. 2014, p.1-18, 2014.

Trytek, M.; Fiedurek, J. Biotransformation of d-limonene to carvone by means of glucose oxidase and peroxidase. **Acta Biochimica Polonica**, v. 51, n. 1, p. 57-62, 2002.

Udayanga, D.; Liu, X.; McKenzie, E. H. C.; Chukeatirote, E.; Ali H. A.; Bahkali, A. H. A.; Hyde, K. D. The genus *Phomopsis*: biology, applications, species concepts and names of common phytopathogens. **Fungal Diversity**, v. 50, n. 1, p.198-225, 2011.

United States Department of Agriculture. Citrus: World Markets and Trade. 2016. Available in: <https://apps.fas.usda.gov/psdonline/circulars/citrus.pdf>, Accessed in: 23/09/2016.

United States Department of Agriculture. (2017) Citrus: World Markets and Trade. Available in: <https://apps.fas.usda.gov/psdonline/circulars/citrus.pdf>, Accessed in: 09/03/2017.

Usepa, 1990. Trimmed spearman-karber (TSK) program version 1.5 ecological monitoring research division. Environmental Monitoring Systems Laboratory, USEPA, Cincinnati, OH.

Valente, J.; Zuzarte, M.; Gonçalves, M. J.; Lopes, M. C.; Cavaleiro, C.; Salgueiro, L.; Cruz, M. T.; Antifungal, antioxidant and anti-inflammatory activities of *Oenanthe crocata* L. essential oil. **Food and Chemical Toxicology,** v. 62, p. 349-354, 2013.

Vanek, T.; Valterová, I.; Vanková, R.; Vaisar, T. Biotransformation of (+)-limonene using Solanum aviculare and Dioscorea deltoidea immobilized plant cells. **Biotechnology Letters**, v. 21, p. 625-628, 1999.

Velázquez-Nuñez, M. J.; Avila-Sosa, R.; Palou, E.; López-Malo, A. Antifungal activity of orange (Citrus sinensis var. Valencia) peel essential oil applied by direct addition or vapor contact, Food Control, v. 31, p. 1-4, 2013.

Venugopalan, A.; Srivastava. S. Endophytes as in vitro production platforms of high value plant secondary metabolites. **Biotechnology Advances**, v. 33, n. 6, p. 873–887, 2015.

Villatoro, C.; Altisent, R.; Echeverría, G.; Graell, J.; López, M. L.; Lara, I. Changes in biosynthesis of aroma volatile compounds during on-tree maturation of 'Pink Lady®' apples. **Postharvest Biology and Technology**, v. 47, p. 286-295, 2008.

Viniegra-Gonzàlez, G. Solid state fermentation: Definition, Characteristics, Limitations and Monitoring. In: ROUSSOS, S. LONSANE, B. K. RAIMBAULT, M. VINIEGRA-

GONZÀLEZ, G. **Advances in Solid State Fermentation**. New York: Springer Netherlands, 1997.

Wang, L.; Weller, C. L. Recent advances in extraction of nutraceuticals from plants. **Trends in Food Science & Technology**, v. 17, p. 300-312, 2006.

Wang, Y.; Dai, C-C. Endophytes: a potential resource for biosynthesis, biotransformation, and biodegradation. **Annals of Microbiology**, v. 61, p. 207-215, 2011.

Wicklow, D. T.; Shotwell, O. L. 1983. Intrafungal distribution of aflatoxin among conidia and esclerotia of *Aspergillus flavus* and *Aspergillus parasiticus*. **Canadian Journal of Microbiology**, v. 29, p. 1-5, 1983.

Wong, W. S.; McLean, A. E. Effects of phenolic antioxidants and flavonoids on DNA synthesis in rat liver, spleen, and testis in vitro. **Toxicology**, v. 139, p. 243–253, 1999.

Woo, C.; W. Man, R. Y.; Siow, Y. L.; Choy, P. C.; Wan, E. W.; Lau, C. S. Ganoderma lucidum inhibits inducible nitric oxide synthase expression in macrophages. **Molecular and Cellular Biochemistry**, v. 275, p.165-171, 2005.

Xie, X-G. ; Huang, C-Y. ; Fu, W-Q. ; Dai, C-C. Potential of endophytic fungus *Phomopsis liquidambari* for transformation and degradation of recalcitrant pollutant sinapic acid. **Fungal Biology**, v. 120, p. 402-413, 2016.

Yadava, A. R.; Chauhan, A. S.; Rekha, M. N.; Ramteke, R. S. Flavour quality of dehydrated lime [Citrus aurantifolia (Christm.) Swingle]. **Food Chemistry**, v. 85, p. 59-62, 2004.

Yang, F-C.; Ma, T-W.; Lee, Y-H. Reuse of citrus peel to enhance the formation of bioactive metabolite-triterpenoid in solid-state fermentation of *A. cinnamomea*. **Biochemical Engineering Journal**, v. 78, p. 59–66, 2013.

Yang, Y.; Kayan, B.; Neval, B.; Pate, B.; C. Baker, C.; Gizir, A. M. Terpene degradation and extraction from basil and oregano leaves using subcritical water. **Journal of Cromatography A**, v. 1152, p. 262-267, 2007.

Yoo, S. K; Day, D. F. Bacterial metabolism of and -pinene and related monoterpenes by Pseudomonas sp. strain PIN. **Process Biotechnology**, v. 37, p. 739-745, 2002.

Zeng, W. L.; Li, W. K.; Han, H.; Tao, Y. Y.; Yang, L.; Wang, Z. T.; Chen, K. X. Microbial biotransformation of gentiopicroside by the endophytic fungus *Penicillium*

crustosum 2T01Y01. **Applied and Environmental Microbiology**, v. 80, p. 184-192, 2014.

Zhang, Y. ; Shi, J. ; Gao, Y. ; Yangwu, R. ; Jiang, H.; Che, J. ; Liu, Y. Production of pinoresinol diglucoside, pinoresinol monoglucoside, and pinoresinol by *Phomopsis* sp. XP-8 using mung bean and its major components. **Applied Microbiology and Biotechnology**, v. 99, p. 4629-4643, 2015.

Zhao, S.; Zhang, D. Supercritical CO2 extraction of Eucalyptus leaves oil and comparison with Soxhlet extraction and hydro-distillation methods. **Separation and Purification Technology,** v. 133, p. 443-451, 2014.

Živković, S, Jošić, D, Popović, T, 2012. Characterizantion of *Diaporthe/Phomopsis* spp. from plum trees by dds-page. In: International symposium on current trends in plant protection, Belgrade, Sérvia.

Zulueta, A.; Esteve, M. J.; Frígola, A. ORAC and TEAC assays comparison to measure the antioxidant capacity of food products. **Food Chemistry**, v. 114, p. 310-316, 2009.

Zurier, R. B. Reduction of Phagocytosis and Lysosomal Enzyme Release From Human Leukocytes by Serum from Patients With Systemic Lupus Erythematosus. **Arthritis and Rheumatism**, v. 19, n. 1, p. 73-78, 1976.